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A QUANTITATIVE COMPARISON OF CASEIN, LACTALBUMIN, AND EDESTIN FOR GROWTH OR MAINTENANCE.*

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Our earliest attempts[1] to make quantitative comparisons of the nutritive values of different proteins for either growth or maintenance were subject to the criticism that the food intake was not sufficiently controlled to justify positive conclusions unless the nutritive differences between the proteins under investigation were very pronounced. The data furnished by the former trials in which the size of the animal and the food intakes were fairly comparable indicated, however, that not only were these proteins unlike in their efficiency when used in the same proportion as supplements to an inadequate protein like zein,[2] but the rate of growth on foods containing the same percentages of these proteins was quite unlike. As food was offered *ad libitum* to the animals it rarely happened that the total calorie intake, the absolute amount of protein eaten, the quantities of inorganic salts, or amount of "food accessories," were strictly comparable.

We have endeavored therefore to plan our latest experiments so as to meet all criticisms of this sort. When animals grow they require more nutriment, owing to the increased amount of tissue formed. Obviously the efficiency of a diet for an animal which grows cannot be compared quantitatively with that of a diet upon which an animal makes little, if any, growth. The

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351; 1915, xxii, 241.

[2] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325.

question may fairly be asked: Does the one animal grow because it eats more or the other fail to grow because it eats less? Definite conclusions can only be drawn from experiments where, during the same period of time, substantially the same amount of food was eaten by animals of corresponding size. Such conditions can be established only by feeding weighed quantities of food in amounts so chosen that the animal will consume all that is offered each day.

In order to learn the minimal amount of food needed for a normal rate of growth several young rats were fed on weighed quantities of a ration of the isolated food materials which, in our earliest experiments, we had fed *ad libitum*. By increasing or decreasing, from day to day, the quantity of food offered, according as the weight of the animal fell below or above the normal, the growth was so controlled as to keep all of the animals growing at very nearly the same rate. Little difficulty was encountered in inducing the animals to eat all of the food offered, since the amount supplied was somewhat below that ordinarily consumed by them when fed *ad libitum*. On the following diet:

	per cent
Lactalbumin	18
"Protein-free milk"	28
Starch	28
Butter fat	18
Lard	8

the weekly intake in the case of the two males, started at essentially the same age and size, thus limited daily in quantity, was as follows:

Normal Growth, Limited Daily Feeding.

Week of limited feeding.	Rat 2586.		Rat 2593.		Average food intake per week.
	Food intake per week.	Gain in body weight.	Food intake per week.	Gain in body weight.	
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
1	22.9	8	25.5	9	24.2
2	39.0	20	40.0	20	39.5
3	39.5	15	44.5	12	42.0
4	48.0	17	45.0	16	46.5
5	49.5	9	51.0	15	50.3
Total	198.9	69	206.0	72	202.5

Inasmuch as excess of protein fed does not increase the rate of growth beyond the normal limits "constitutionally fixed" for the individual it seemed desirable, in instituting any careful comparison of the physiological value of different proteins, to avoid, if possible, feeding even the slightest excess above the minimum actually required for so called normal growth. It was therefore planned to imitate the preliminary experiments just referred to—which were carried out with a protein, lactalbumin, that had already given some justification for the belief in its superior nutrient value—by trials with limited daily food intakes precisely alike in energy content, inorganic and accessory food ingredients, etc., for each day, in Series A.

Series A.—This series was to be varied in respect to the protein added. The food intake was uniformly about 10 per cent lower than that in the preliminary series, so that in any event there would be no unnecessary surplus of food ingested. The rats —all males—were of approximately the same body weight (45 to 50 gm.) at the beginning of the experiment. With conditions thus favorably selected, so that the diet would barely suffice for growth at a normal rate when the protein employed was as efficient as the lactalbumin which had served as the guide for the quantities needed, further trials were begun with edestin and with casein, alone or with the addition of the amino-acids cystine or alanine. The period of comparison in this series lasted 77 days. The animals, almost without exception, consumed all of the food offered to them daily, so that the total intake for the entire period in each of the large number of experiments undertaken scarcely varied more than 2 or 3 gm. Whenever an animal showed any abnormality, such as a continued failure to eat, it was discarded; and accordingly no data from such aberrant individuals are included in the statistical averages which are tabulated in Table I. This shows the initial weights of the rats, the food intakes in the successive weeks of the entire period, and the total gains made in body weight up to the end of each successive week. In view of the fairly comparable character of the results obtained with different individuals in the same series of the trials, it has seemed justifiable to compare weekly averages for each group. These are summarized in Table II. The composition of the foods was as follows:

	per cent
Protein	8.0–20.45
"Protein-free milk"	28
Starch / Sucrose	36–23
Butter fat	18
Lard	7–12

When amino-acids were fed with casein they represented an addition equivalent to 3 per cent of the added protein.

An inspection of these statistics will show that in animals of the same sex, with the same initial body weight, and the same total food intake furnished daily in precisely equivalent and increasing amounts in successive periods of the experiment, in accord with the anticipated needs of increasing body weight, *the lactalbumin foods in every case produced the largest gains.* For example, the average total gain in 11 weeks upon the diet containing 14.8 per cent of lactalbumin was 122 gm., in contrast with the gain of 105 gm. and 95 gm. on similar diets of 16.2 per cent of casein and 16.7 per cent of edestin respectively. Even with larger percentages of these proteins (casein 19.8 per cent and edestin 20.5 per cent) the total gains are below that obtained on a smaller daily intake of protein in the form of lactalbumin. The highest concentrations of other proteins here recorded likewise have failed to exceed to any significant extent the average total gain with lactalbumin foods containing only 9.9 per cent of this protein.

In view of the fact that the food intakes were strictly comparable in these experiments, and that the trials were extended over a very long period during which temporary remissions or accelerations of growth might easily correct themselves, *the superior efficiency of lactalbumin in the nutrition of growth is demonstrated* beyond question. Incidentally it should be noted that inasmuch as the animals were all receiving the same absolute amounts of food on the corresponding day of each period, the rapidly growing animals were actually put at a *disadvantage* in having a smaller allowance of food *per unit of body weight* as the result of the superior growth-promoting qualities of the protein ingested.

We have intimated elsewhere[3] that the comparative inferiority of casein foods in producing growth when smaller amounts, *e.g.*,

[3] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351.

9 per cent, of the protein are furnished in the ration, can be corrected by the addition of the essential amino-acid cystine which this protein yields in relatively small amounts as compared with most other proteins. Our newer experiments in which the casein was supplemented by sufficient cystine to make the total available quantity of this amino-acid equivalent to that furnished by many other proteins, likewise show that the problem of the comparative nutritive value of smaller quantities of casein is largely, if not entirely, concerned with this factor; thus, whereas with a diet containing 8 per cent of casein the total gain during the 11 weeks was only 71 gm., in contrast with 77 gm. gained on the food containing 8 per cent of lactalbumin, the addition of cystine to the same amount of casein enabled the individuals to utilize this protein for growth quite as efficiently, while the experiments lasted, as they did the corresponding quantity of lactalbumin. This is a specific result—not merely one due to some stimulating feature of amino-acids in general; for the addition of the same amount of alanine under precisely similar conditions failed to induce more rapid growth.

It seems unnecessary to reproduce here all the numerous details of these rather laborious experiments. The results are graphically presented in the form of composite curves in the appendix, Chart I. This shows in a striking way the nutrient disadvantages of too low concentrations of protein in the diet, although in view of the unlike physiological value of the different proteins ingested, a single absolute minimum intake value for a definite intensity of growth cannot be made to apply equally to all of them.[4]

Our experiments show further that exhibition of protein beyond

[4] Obviously the unlike activity of the individual rats introduces a variable in respect to energy requirement which is beyond immediate control. This may account for the failure of many of the animals to make suitable gains in weight during the 1st week of the feeding trials. It may be that this was occasioned by exceptional activity, leading to unusual transformation of energy, as an expression of restlessness due to the conditions of limited feeding to which the animals had not yet become accustomed. According to the experience which we have presented elsewhere (*Am. J. Physiol.*, 1916, xl, 16) it may perhaps be assumed that the acceleration of growth following these temporary retardations would ultimately equalize the temporary failures to grow.

TABLE I.

*Series A. Statistics of Gains in Body Weight after Uniform Daily Intakes of Food.**

Protein.	Rat.	Days.	7	14	21	28	35	42	49	56	63	70	77
		Total food, gm.	23	53	88	126	165	207	249	291	340	389	438
		Initial weight.	Gain.										
per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Casein 19.8	2773	45.5	5.5	18.0	32.5	37.5	48.5	57.0	64.5	70.0	82.0	90.5	95.5
	2779	48.5	9.0	23.5	35.5	39.0	51.0	63.0	68.5	76.0	88.0	97.5	104.0
	2792	47.0	3.5	17.5	28.0	34.0	46.0	55.0	62.0	71.5	88.0	98.0	100.0
Edestin 20.5	2794	47.0	7.5	18.0	26.0	35.0	43.0	48.5	54.5	68.0	80.0	90.0	99.0
	2795	46.5	5.0	15.0	21.0	29.5	41.0	52.0	61.0	74.0	88.5	94.5	105.5
	2800	49.0	5.0	14.0	20.0	30.5	36.0	48.0	55.5	66.0	80.0	87.5	98.0
Lactalbumin 14.8	2645	44.5	8.0	23.0	37.0	49.0	60.0	73.5	82.5	89.0	106.0	114.0	124.5
	2646	50.5	10.0	16.5	30.0	41.0	55.5	66.5	76.0	81.0	94.5	108.5	119.0
Casein 16.2	2727	49.0	6.0	12.0	24.0	28.0	43.0	60.5	67.5	Died.			
	2733	44.0	6.0	15.0	28.0	37.0	54.0	67.5	74.5	85.0	97.0	99.0	109.0
	3308	48.0	3.0	10.5	15.0	25.0	34.0	47.0	59.0	67.0	79.0	93.0	102.0
	3322	45.0	6.0	15.0	15.0	16.5	31.5	48.0	54.5	63.0	76.0	92.5	107.5
	3383	47.5	1.5	9.5	20.0	32.0	41.5	55.5	63.5	70.0	87.5	99.5	101.0
Edestin 16.7	2749	48.0	2.0	13.0	24.5	35.5	46.0	59.0	69.0	72.5	85.0	93.0	97.0
	2751	45.0	4.0	14.0	25.0	36.0	48.0	60.0	67.5	67.5	80.0	88.5	92.0
Lactalbumin 9.9	2772	43.5	5.0	14.5	26.0	35.5	41.5	48.5	58.0	67.5	80.0	89.5	98.5
	2782	45.5	9.0	21.5	34.5	41.5	54.0	61.0	65.5	72.5	82.0	92.0	95.5
	2786	48.0	7.5	19.5	31.0	39.0	49.5	57.5	65.0	74.5	91.0	100.0	107.0

Casein 10.8	2828	49.5	2.0	8.0	19.0	30.0	34.0	44.0	53.5	59.0	71.5	82.0	89.5
	2830	47.5	2.5	7.5	24.0	32.0	37.5	45.0	54.5	62.5	73.5	84.0	81.0
	2831	51.0	−3.0	1.5	12.5	22.0	30.0	37.0	42.0	46.5	56.5	67.0	Discontinued.
Edestin 11.2	2829	49.5	2.0	6.5	19.5	25.0	30.5	39.5	47.5	51.5	59.0	69.0	80.0
	2832	53.5	−2.0	3.5	12.5	19.0	25.5	32.5	38.0	42.5	49.0	55.0	Discontinued.
	2833	48.5	2.0	7.0	17.5	24.0	30.0	37.0	44.5	50.0	59.5	66.0	74.5
Lactalbumin 8	3192	45.0	−1.5	5.5	16.0	24.0	34.0	42.0	51.0	60.0	71.0	78.0	85.5
	3193	54.0	−3.0	4.5	14.0	23.0	28.0	35.0	41.0	49.5	53.0	56.5	60.0
	3194	47.0	−5.0	7.0	17.0	26.0	33.5	41.0	52.0	60.5	70.0	78.0	84.0
Casein 8 + cystine	3307	46.5	2.5	8.0	17.0	26.5	31.5	42.0	50.5	58.5	71.5	88.5	94.5
	3315	45.5	5.5	13.0	18.0	23.5	30.0	Died.					
	3321	46.0	2.0	9.0	15.0	24.0	37.0	50.0	56.5	60.0	65.0	Died.	
Casein 8 + alanine	3345	45.0	2.5	4.0	9.0	16.5	22.0	26.0	31.5	39.5	Discontinued.		
	3346	45.5	0.0	1.5	8.0	16.5	19.5	28.0	38.5	44.5	52.5	Discontinued.	
	3353	46.5	−2.5	2.0	9.0	17.0	21.5	30.0	35.0	40.5	49.0	55.5	62.5
Casein 8	3206	46.5	1.0	5.5	15.5	23.5	28.0	36.5	46.5	52.5	60.0	65.5	69.0
	3207	47.0	0.5	6.0	14.0	22.0	27.0	34.5	44.0	48.5	55.5	64.0	71.0
	3216	48.0	2.5	11.0	21.0	24.0	28.0	34.5	43.0	49.5	59.5	66.0	73 0
Edestin 8	3210	46.0	0.0	5.0	10.5	17.0	21.0	23.5	29.5	33.5	40.0	46.0	52.5
	3212	45.0	0.0	6.0	10.0	16.0	23.0	23.5	30.0	35.5	43.0	47.5	57.0
	3347	47.5	−10.0	−8.0	2.0	12.0	19.0	24.0	24.5	29.0	33.5	38.0	40.5

* The figures for protein do not include the small amount of protein present in the "protein-free milk." For a discussion of this see a previous paper.[5]

TABLE II.

*Series A. Summary of Averages from Table I.**

Protein.	Days.	7		14		21		28		35		42		49		56		63		70		77	
	Total food, gm.	23		53		88		126		165		207		249		291		340		389		438	
	Initial weight.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.	Total protein.	Total gain.
per cent	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Casein 19.8.....	47.0	4.6	6	10.5	20	17.5	32	25.0	37	32.6	49	41.0	58	49.4	65	57.6	73	67.3	86	77.0	95	86.7	100
Edestin 20.5.....	47.5	4.8	6	10.9	16	18.1	22	25.9	32	33.8	40	42.4	50	51.1	57	59.6	69	69.6	83	79.6	91	89.8	101
Lactalbumin 14.8.	47.5	3.4	9	7.9	20	13.0	34	18.7	45	24.4	58	30.6	70	36.8	79	43.1	85	50.3	100	57.5	111	64.8	122
Casein 16.2.....	46.7	3.8	5	8.6	12	14.3	20	20.5	28	26.7	41	33.6	56	40.4	64	47.2	71	55.2	85	63.0	96	71.0	105
Edestin 16.7.....	46.5	3.9	3	8.9	14	14.7	25	21.1	36	27.5	47	34.6	60	41.6	68	48.6	70	56.7	83	65.0	91	73.1	95
Lactalbumin 9.9..	45.7	2.3	7	5.2	19	8.7	31	12.6	39	16.3	48	20.5	56	24.6	63	28.8	72	33.6	84	38.5	94	43.3	100
Casein 10.8.....	49.3	2.5	1	5.7	6	9.5	19	13.8	28	17.8	34	22.4	42	26.9	50	31.4	56	36.7	67	42.0	78	47.3	85
Edestin 11.2.....	50.5	2.6	1	5.9	6	9.9	17	14.2	23	18.5	29	23.2	36	27.9	43	32.6	48	38.1	56	43.5	63	49.0	77
Lactalbumin 8....	48.7	1.9	−3	4.2	6	7.0	16	10.1	24	13.2	32	16.6	39	19.9	48	23.3	57	27.2	65	31.1	71	35.0	77
Casein 8+cystine.	46.0	1.9	3	4.2	10	7.0	17	10.1	25	13.2	33	16.6	46	19.9	54	23.3	59	27.2	68	31.1	89	35.0	95
" 8+alanine.	45.7	1.9	0	4.2	3	7.0	9	10.1	17	13.2	21	16.6	28	19.9	35	23.3	42	27.2	51	31.1	56	35.0	63
" 8.....	47.2	1.9	1	4.2	8	7.0	17	10.1	23	13.2	28	16.6	35	19.9	44	23.3	50	27.2	58	31.1	65	35.0	71
Edestin 8.....	46.4	1.9	−3	4.2	1	7.0	8	10.1	15	13.2	21	16.6	24	19.9	28	23.3	33	27.2	39	31.1	44	35.0	50

* The figures for protein do not include the small amount of protein present in the "protein-free milk." For a discussion of this see a previous paper.[5]

a certain concentration, which we have found to be approximately 12.5 per cent of the total calories, fails to increase the gains in body weight. The rats which received 19.8 and 20.5 per cent of casein and edestin respectively in their diet have not surpassed in growth those receiving food with only 16.2 and 16.7 per cent of the same proteins.

Series B.—In the series of experiments described in the preceding section the food intake of each animal was increased to precisely the same degree at the same successive interval, independent of whether the gains of weight in the different subjects were alike or not. The criticism might, therefore, properly be made that in view of the unlike rate of growth upon the same *absolute* intake of the different foods the larger animals were put at a nutrient disadvantage from the quantitative standpoint. Accordingly the actual quantitative differences between the physiological values of the different proteins would fail to be revealed to their fullest extent. The only strict basis for comparison is afforded by experiments in which the animals receive the same amount of food during the same period of time and make the same gain in weight.

Guided by our past experience we endeavored so to plan our experiments that these conditions would be fulfilled. The total food intake was increased systematically in harmony with the growth of each subject. Male rats were used. The weighed quantities of food supplied at successively increasing sizes (as indicated by body weights) were equivalent in calories to the intake determined by statistics of animals growing at a normal rate and receiving *ad libitum* a ration of essentially the same composition and energy value as those recorded in the present study.[3]

In order to eliminate, as far as possible, inequalities in the rate of growth of different individuals and also to be certain that no protein would be "wasted" by feeding more than the individual could actually utilize in the metabolism of growth, the concentration of *protein* in the rations of the present series was lowered so that with a sufficient total *energy* intake, the protein furnished was insufficient to permit average normal growth. Here again lactalbumin was taken as a standard with which edestin and casein (alone or with cystine or alanine) could be compared. As might

be anticipated, under these conditions of nutrition, identical in all respects except as regards the quantity of protein eaten, lactalbumin has proved itself to be quantitatively superior to both casein and edestin. *Thus, to produce the same gain in body weight 50 per cent more casein than lactalbumin was required, and of edestin nearly 90 per cent more.*

To demonstrate that in these experiments the protein was the limiting factor, comparison should be made with those experiments in which smaller quantities of casein or edestin were supplied (see Chart III). It is seen that even when the amount of these proteins considerably exceeded that of the lactalbumin, growth proceeded at a distinctly slower rate, although at corresponding sizes the same quantity of food and a relatively larger quantity of protein than of the lactalbumin was consumed.

In order to overcome inequalities in the rate of growth and food consumption, we have attempted to ascertain for edestin and casein how much the protein content of the ration in each of these respective cases needed to be increased in order to insure a rate of growth precisely equal to that upon our "standard" lactalbumin food containing 8 per cent of the added protein. Obviously when animals make the same gain of weight in the same period of time, and receive equivalent rations during each successive gain in weight, their growth will be accomplished through the same expenditure of energy and with the same intake of inorganic salts and "food accessories," the sole variable being the content of protein. This was accomplished by the use of approximately 12 per cent of casein and 15 per cent of edestin respectively. The same weight was gained as when 8 per cent of lactalbumin was used (see Chart II). The variations in the content of protein in a ration of otherwise constant energy value were made possible by substitutions of starch for protein and *vice versa* in our standard food mixture, thus not altering essentially their calorific value. The resulting make-up of the rations fed is given below.

	Protein (per cent).					
	8	8 (+ amino-acid).	10	12	13.5	15
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Protein	8	8.00	10	12	13.5	15
Amino-acid	0	0.24	0	0	0.0	0
Starch and moisture	36	35.76	34	32	30.5	29
"Protein-free milk"	28	28.00	28	28	28.0	28
Butter fat	18	18.00	18	18	18.0	18
Lard	10	10.00	10	10	10.0	10

With the lower concentrations of the other proteins used the rate of growth was in every case noticeably behind that produced by the standard lactalbumin food.

Chart II shows the composite graphs of the curves of rats receiving, in daily limited amounts, a food mixture containing 8 per cent of lactalbumin, 12 per cent of casein, or 15 per cent of edestin as its essential nitrogenous component. The results of the experiments which they represent are strictly comparable. Chart III shows the composite graphs of curves of rats similarly receiving a food mixture containing smaller proportions of casein or edestin in comparison with 8 per cent of lactalbumin. One of the curves shows the result of furnishing a larger proportion of casein (13.5 per cent).

The animals were weighed daily and were fed on the special diets when they reached a size of approximately 80 gm. During the first 3 days 5.7 gm. of food per day were given; they then received 5.8 gm. per day until a body weight of 95 gm. was attained. Thereupon the daily limited intake of food was increased after each 5 gm. of gain in weight according to the following scheme:

Body weight.	Daily food intake.	Body weight.	Daily food intake.
gm.	*gm.*	*gm.*	*gm.*
95–100	6.1	140–145	8.3
100–105	6.4	145–150	8.5
105–110	6.7	150–155	8.7
110–115	7.1	155–160	8.9
115–120	7.3	160–165	9.1
120–125	7.6	165–170	9.3
125–130	7.9	170–175	9.5
130–135	8.0	175–180	9.7
135–140	8.1		

TABLE III.

*Series B. Statistics of Food Intake and Gains in Body Weight with Comparative Intakes of Different Proteins.**

Protein.	Days.	7		14		21		28		35		42		49		56	
	Rat.	Food intake.	Gain.	Food intake.	Gain.	Food intake.	Gain.	Food intake.	Gain.	Food intake.	Gain.	Food intake.	Gain.	Food intake.	Gain.	Food intake.	Gain.
per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Lactalbumin 8	2986	39.7	8.5	81.8	17.0	125.7	22.5	173.2	35.0	226.5	48.5	283.3	60.0	342.2	72.0	405.3	87.0
	3000	40.3	1.0	80.9	5.5	122.1	17.0	168.5	28.5	219.8	40.5	275.2	50.5	333.1	62.5	394.2	81.0
	3018	40.3	3.5	80.9	12.0	125.3	22.5	174.2	35.5	227.1	45.5	283.4	59.5	342.9	73.5	400.8	88.5
Casein 13.5	3065	40.3	7.5	81.5	14.5	128.0	31.5	179.5	45.5	235.4	59.5	294.5	77.0	358.6	92.5	426.5	106.0
	3080	40.3	5.0	80.9	12.0	124.5	19.5	171.2	29.5	223.1	43.5	278.9	55.0	335.7	67.5	397.4	78.0
Casein 12	3026	40.3	6.0	80.9	15.0	124.8	30.0	175.2	46.5	231.0	56.5	288.7	66.0	349.4	83.0	414.1	99.0
	3038	40.3	1.5	79.1	6.0	123.6	24.0	172.9	37.0	226.9	49.0	283.8	62.0	343.7	78.5	408.8	95.5
	3077	40.3	7.5	80.9	13.5	124.5	23.0	172.3	34.5	225.5	48.5	282.0	62.0	341.5	77.0	405.2	93.5
Casein 10	3109	40.6	5.5	82.4	12.0	126.9	17.0	174.7	28.0	227.1	40.0	283.1	50.0	341.4	62.0	402.9	72.0
	3111	40.6	5.5	81.2	9.5	123.3	14.0	167.2	25.0	217.1	39.0	272.1	44.0	328.2	52.0	385.9	60.0
	3119	40.3	2.0	80.9	4.5	121.5	9.0	162.1	16.5	206.9	28.5	256.8	38.5	310.6	52.0	367.1	61.0
Casein 8 + cystine	3176	40.3	4.5	80.9	11.5	123.3	18.0	169.0	26.5	219.5	41.5	274.7	54.5	332.2	64.5	392.3	70.5
	3255	40.3	1.0	80.9	11.0	122.4	22.0	169.5	37.0	220.6	42.5	273.8	49.5	329.9	62.0	388.2	69.5
	3318	40.3	4.0	80.9	6.5	121.5	10.5	163.6	22.0	212.4	38.0	266.5	51.0	322.9	62.0	382.4	76.0

Casein 8 + alanine	3372	40.3	−2.0	80.9	−4.0	121.5	2.0	162.1	8.0	204.5	18.5	252.0	28.5	302.7	35.0	357.1	43.0
	3373	40.3	0.5	80.9	2.0	121.5	2.0	162.7	21.0	209.0	31.5	260.1	44.0	313.8	50.0	372.4	52.0
	3379	40.3	−2.5	80.9	7.5	121.5	14.0	164.2	21.0	211.6	32.0	259.7	39.0	306.3	41.5	359.0	45.5
Casein 8	3301	40.3	1.0	80.9	2.0	121.5	4.0	162.1	10.0	202.7	12.0	245.4	22.0	292.1	30.0	338.2	35.0
	3302	40.3	−1.0	80.9	1.0	121.5	2.0	162.1	3.5	203.0	10.5	246.9	22.0	296.0	32.5	347.7	37.0
	3303	40.3	1.0	80.9	3.5	121.5	7.0	162.1	10.5	203.6	17.0	247.5	26.0	291.3	31.5	330.3	34.0
	3317	40.3	−1.0	80.9	1.0	121.5	4.0	162.1	14.0	205.1	20.0	250.5	27.0	299.4	34.5	351.4	45.0
Edestin 15	3125	40.3	2.0	80.9	13.0	125.4	23.5	174.1	35.5	228.2	50.0	285.2	61.5	345.7	77.5	410.8	94.5
	3126	40.3	6.5	80.9	11.5	122.4	19.5	168.2	32.0	220.1	42.5	275.0	53.0	332.2	65.0	393.1	81.0
	3228	40.3	3.0	80.9	7.5	122.1	17.5	167.8	31.0	219.6	46.0	275.6	57.0	324.5	70.0	397.0	83.0
	3282	40.3	5.0	80.9	12.5	123.0	22.0	168.7	31.0	220.0	43.0	275.4	57.0	333.5	73.0	396.2	87.0
	3283	40.3	3.0	80.9	11.0	123.0	21.0	169.4	30.0	220.5	44.5	276.4	59.5	335.7	76.5	399.0	87.5
Edestin 12	2997	40.3	1.0	80.9	5.0	122.7	12.0	168.1	19.0	217.6	30.0	271.4	40.0	327.6	52.0	386.5	62.0
	3045	40.3	1.0	80.9	8.0	121.5	11.0	165.1	20.0	213.6	32.0	265.6	44.0	321.4	54.0	379.3	63.0
	3070	40.3	5.0	80.9	12.0	122.1	20.0	167.2	29.0	217.1	39.0	270.9	51.0	327.4	63.0	385.1	67.0

* The figures for protein do not include the small amount of protein present in the "protein-free milk." For a discussion of this see a previous paper.[5]

TABLE IV.

*Series B. Summary of Averages from Table III.**

Days.	7			14			21			28			35			42			49			56		
Protein.	Total food intake.	Total protein intake.	Total gain.	Total food intake.	Total protein intake.	Total gain.	Total food intake.	Total protein intake.	Total gain.	Total food intake.	Total protein intake.	Total gain.	Total food intake.	Total protein intake.	Total gain.	Total food intake.	Total protein intake.	Total gain.	Total food intake.	Total protein intake.	Total gain.	Total food intake.	Total protein intake.	Total gain.
per cent	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Lactalbumin 8....	40	3.2	4	81	6.5	12	124	9.9	21	172	13.8	33	225	18.0	45	281	22.5	57	339	27.2	69	400	32.0	86
Casein 13.5.....	40	5.4	6	81	11.0	13	127	17.1	26	175	23.7	38	229	31.0	52	287	38.8	66	347	46.9	80	412	55.6	92
" 12.....	40	4.8	5	80	9.6	12	124	14.9	26	174	20.8	39	228	27.3	51	285	34.2	63	344	41.3	80	410	49.2	96
" 10.....	40	4.1	4	82	8.2	9	124	12.4	13	168	16.8	23	217	21.7	36	271	27.1	43	327	32.7	55	385	38.5	64
" 8+cystine.	40	3.2	3	81	6.5	10	122	9.8	17	167	13.4	29	218	17.4	41	272	21.7	52	328	26.3	63	388	31.0	72
" 8+alanine.	40	3.2	−1	81	6.5	2	122	9.7	9	163	13.0	17	208	16.7	27	257	20.6	37	308	24.6	42	363	29.0	47
" 8.....	40	3.2	0	81	6.5	2	122	9.7	4	162	13.0	10	204	16.3	15	248	19.8	24	295	23.6	32	346	27.6	39
Edestin 15.....	40	6.0	4	81	12.1	11	123	18.5	21	170	25.4	32	222	33.3	45	278	41.6	56	336	50.5	72	399	59.9	87
" 12.....	40	4.8	2	81	9.7	8	122	14.7	14	167	20.0	23	216	25.9	34	269	32.3	45	326	39.1	56	384	46.1	64

* The figures for protein do not include the small amount of protein present in the "protein-free milk." For a discussion of this see a previous paper.[5]

The detailed statistics are recorded in Table III, the weekly averages being summarized in Table IV. These show the intakes of food and protein as well as the gain in body weight totalled to the end of each successive week. The experiments of this series lasted 56 days.

An inspection of Table IV shows that the essentially equal gains made by the rats receiving 8 per cent of lactalbumin, 12 per cent of casein, and 15 per cent of edestin respectively were made with approximately equal intakes of food, namely: lactalbumin 8 per cent, 400 gm.; edestin 15 per cent, 399 gm.; casein 12 per cent, 410 gm. The diet and growth in these groups are strictly comparable, yet the total intakes of added protein to accomplish these equivalent gains in the three cases average 32.0, 59.9, and 49.2 gm. respectively. This is the most conclusive evidence which we have yet obtained of the unequal value of different proteins in growth.

In this series, as in Series A, the addition of cystine, equivalent to 3 per cent of the casein used, effected considerable economy in the availability of this protein. Thus the average results of three 8 week experiments are compared below.

Protein in food.	Total gain in weight.	Food intake.	Protein intake.
	gm.	*gm.*	*gm.*
Casein 10 per cent	64	385	38.5
" 8 " " + cystine	72	388	31.0

We thus see that the addition of cystine rendered the casein so much more efficient for growth that, on the average, 18 per cent *less* protein produced 12.5 per cent more growth. This capacity of cystine to supplement casein and thus increase its nutritive value is specific, depending on the chemical constitution of the amino-acid. It has been suggested that some amino-acids, such as alanine, may stimulate metabolism, thus promoting the food intake of the animal. The latter possibility has been prevented in our experiments by the control of the food supply. The replacement of cystine by alanine fails to bring about any nutritive advantage. (See Tables III and IV, casein 8 per cent + alanine.)

A comparison of the effect of supplements of alanine to the 8 per cent casein food is shown in Chart III and the tabular summaries.

Comparative Physiological Efficiency of Different Proteins in Maintenance without Growth.

When growth is not involved the requirement of protein is smaller. In consequence, inequalities between the different proteins are likely to manifest themselves less conspicuously than when continued increments of body weight augment the demand for nitrogenous foodstuffs. As the result of preliminary experiments on the protein minima for maintenance, it was stated:

"Ranges of 7 to 15 mg. of lactalbumin per gm. of rat per week represent minima lower than those found for casein, edestin, milk proteins, or gliadin, not to mention the impossibility of maintenance with zein. With the pronounced individual variations in the total food intake exhibited in our experience it is almost impossible to make precise comparisons of rats of the same size on precisely the same intake of protein. It is an obvious shortcoming of this method of procedure that the energy intake is not controlled, so that when it is large there may be a relatively greater protein-sparing effect where the non-protein nutrients are ingested in undue amounts."[5]

In order to meet objections which might arise, particularly because of the unlike daily energy intake of the subjects, further *maintenance* experiments were undertaken in which the daily food intake was limited to a fixed quantity, the protein concentration being adjusted until no essential gain or loss of body weight ensued. The absolute intake of the added protein—either casein, edestin, or lactalbumin—was calculated per gm. of animal per week in order to establish a protein minimum for maintenance in each experiment. Comparisons have been made in this way at different levels of body weight so that the factor of size might be taken into account. The total energy intake was made sufficiently liberal in accord with our experience to have permitted even considerable growth had the supply of protein been sufficient instead of minimal. In addition to the adequacy of the non-protein components of the dietary it will be observed that the inorganic constituents, as well as the food "accessories" were fed

[5] Osborne and Mendel, *J. Biol. Chem.*, 1915, xxii, 253.

in absolutely the same amount daily.[6] The added protein thus represented the chief variable in the experiment aside from such variations as were inevitably induced by the changing body weight of an animal kept on a fixed daily intake of food. Whenever the animals failed to eat satisfactorily, the experiment was terminated. Tables V and VI therefore contain only the statistics from successful trials. The absolute food intake in the case of the trials represented in Table VI being alike in every experiment, the latter series are therefore uniquely comparable. The periods of observation were in all cases sufficiently long to justify a decision regarding a permanent gain or loss of body weight; *i.e.*, whether *prolonged maintenance* on the protein content under consideration was possible.

A few curves are reproduced in Chart IV to illustrate the outcome of this type of experiment. Variations in body weight amounting to no more than 2 or 3 gm. may be regarded as equivalent to a condition of maintenance. It was a common experience to observe that when animals which had been on a diet liberal in protein were fed with a ration sufficiently low in protein to approach the minimum required for maintenance they lost body weight until a sort of nutritive equilibrium was reached at a somewhat lower level. This is exemplified in Rats 3243 and 3272 (Chart IV).

Rats 3110, 3272, and 3096 (Chart IV) illustrate the low level of protein intake on which we have succeeded in maintaining such animals under strictly comparable feeding conditions with the three proteins respectively mentioned. Such observations, as well as the statistics in Tables V and VI, accordingly support our earlier conclusions regarding the comparative superiority of lactalbumin from the standpoint of protein minimum requirement.

[6] The general composition of the food was as follows:

	per cent
"Protein-free milk"	28
Butter fat	18
Lard	7–10
Protein	2.5–5.4
Starch	39–26
Sucrose	0–12

The small amount of protein in the "protein-free milk" has not been taken into account in the calculations. It is essentially the same in the different trials. For a discussion of this feature see a previous paper.[5]

TABLE V.

Protein Minima for Maintenance with Different Proteins.

Rat.	Body weight.	Concentration of protein in the food.*	Total food intake per day.	Food intake per wk. per gm. of rat.	Protein intake per wk. per gm. of rat.*	Gain (+) or loss (−) of body weight during period.	Period of observation.
Lactalbumin.							
	gm.	*per cent*	*gm.*	*mg.*	*mg.*	*gm.*	*days*
2645♂**	170	4.11	7.4	302	12.4	+ 1	42
	167	3.3	7.4	310	10.2	− 5	42
	173	4.95	7.4	302	14.9	+15	28
2646♂	170	4.11	7.4	299	12.3	± 0	42
	165	3.3	7.4	316	10.4	−10	42
	169	4.95	7.4	301	14.9	+18	42
2772♂	130	3.3	7.4	376	12.4	− 1	35
Casein.							
2729♀	126	4.5	6.2	341	15.4	− 1	42
	127	5.4	6.2±	338	18.3	+ 2	42
2732♀	104	4.5	5.2	348	15.7	− 2	28
	102	5.4	5.2	357	19.3	− 1	42
	102	5.04	6.5	440	22.2	+ 1	42
	111	3.33	6.5	412	13.7	− 4	28
	105	3.06	6.5	433	13.2	−11	35
2733♂	137	4.5	6.6	333	15.0	− 6	28
	135	5.4	6.6	340	18.3	+ 2	42
	135	4.5	8.0	414	18.6	− 3	42
	151	3.6	8.0	368	13.2	− 1	21
	150	3.06	8.0	371	11.3	− 1	21
Edestin.							
2749♂	143	4.65	7.0	347	16.2	− 3	42
	142	5.11	7.0	342	17.5	± 0	63
	145	4.46	7.0	343	15.3	+ 5	28
	145	4.18	7.0	333	13.9	− 3	28
	145	3.72	7.0	341	12.7	− 2	28
2751♂**	140	4.65	7.4	380	17.7	+ 6	42
	141	4.18	7.4	369	15.4	− 4	63
	140	4.46	7.0	356	15.9	+ 3	28
	140	3.72	7.0	357	13.3	− 1	28
	142	3.54	7.0	350	12.4	+ 5	35

* This does not include the small amount of protein present in the "protein-free milk." See a previous paper[5] for a discussion of this feature.

** See Chart IV.

TABLE VI.

Protein Minima for Maintenance with Different Proteins.

Rat.	Body weight.	Concentration of protein in the food.*	Total food intake per day.	Food intake per wk. per gm. of rat.	Protein intake per wk. per gm. of rat.*	Gain (+) or loss (−) of body weight during period.	Period of observation.
			Lactalbumin.				
	gm.	*per cent*	*gm.*	*mg.*	*mg.*	*gm.*	*days*
3110 ♀ **	123	3	6	345	10.4	+ 4	28
	129	2.8	6	329	9.2	+ 7	77
	131	2.5	6	321	8.0	− 2	28
3113 ♀	125	3	6	341	10.2	+ 1	28
	126	2.8	6	335	9.4	± 0	77
	126	2.5	6	331	8.3	− 2	28
3115 ♀ **	130	3	6	328	9.8	+ 2	28
	129	2.8	6	328	9.2	− 4	28
	131	3	6±	318	9.5	+ 6	49
			Casein.				
3104 ♀	111	4	6	387	15.5	− 6	35
3199 ♀	109	4	6	387	15.5	− 2	28
3243 ♀ **	119	4	6	373	14.9	± 0	77
3272 ♀ **	111	4	6	381	15.2	− 1	56
	111	3.5	6	383	13.4	− 2	35
3313 ♀	110	4.85	6	386	18.7	+ 7	42
			Edestin.				
3089 ♀	110	4	6±	377	15.1	− 2	28
3096 ♀ **	117	4	6	361	14.5	− 1	21
	117	3.5	6	361	12.6	+ 1	84
	112	3.2	6	373	11.9	−13	35
3097 ♀	112	4	6±	368	14.7	± 0	21
	111	3.5	6±	378	13.3	− 1	84
	107	3.2	6	385	12.4	− 8	35
3197 ♀	104	4	6	400	16.0	+ 1	70
	101	3.5	6	413	14.5	− 6	28
2753 ♀	111	3.72	6	386	14.4	− 1	105
	107	3.26	6	397	13.0	− 9	49
	103	3.72	6	409	15.2	+ 2	21
	103	3.07	6	408	12.5	− 2	28

* This does not include the small amount of protein present in the "protein-free milk." See a previous paper[5] for a discussion of this feature.

** See Chart IV.

We have frequently noted that when the protein concentration of the food becomes very low the animals do not eat satisfactorily, and frequently fail to respond to moderate increments of protein intake which ought promptly to induce renewed growth. Our observations on this apparent physiological damage, possibly attributable to prolonged nutrition on rations of inadequate protein content, need to be extended and confirmed before any generalizations are permissible.

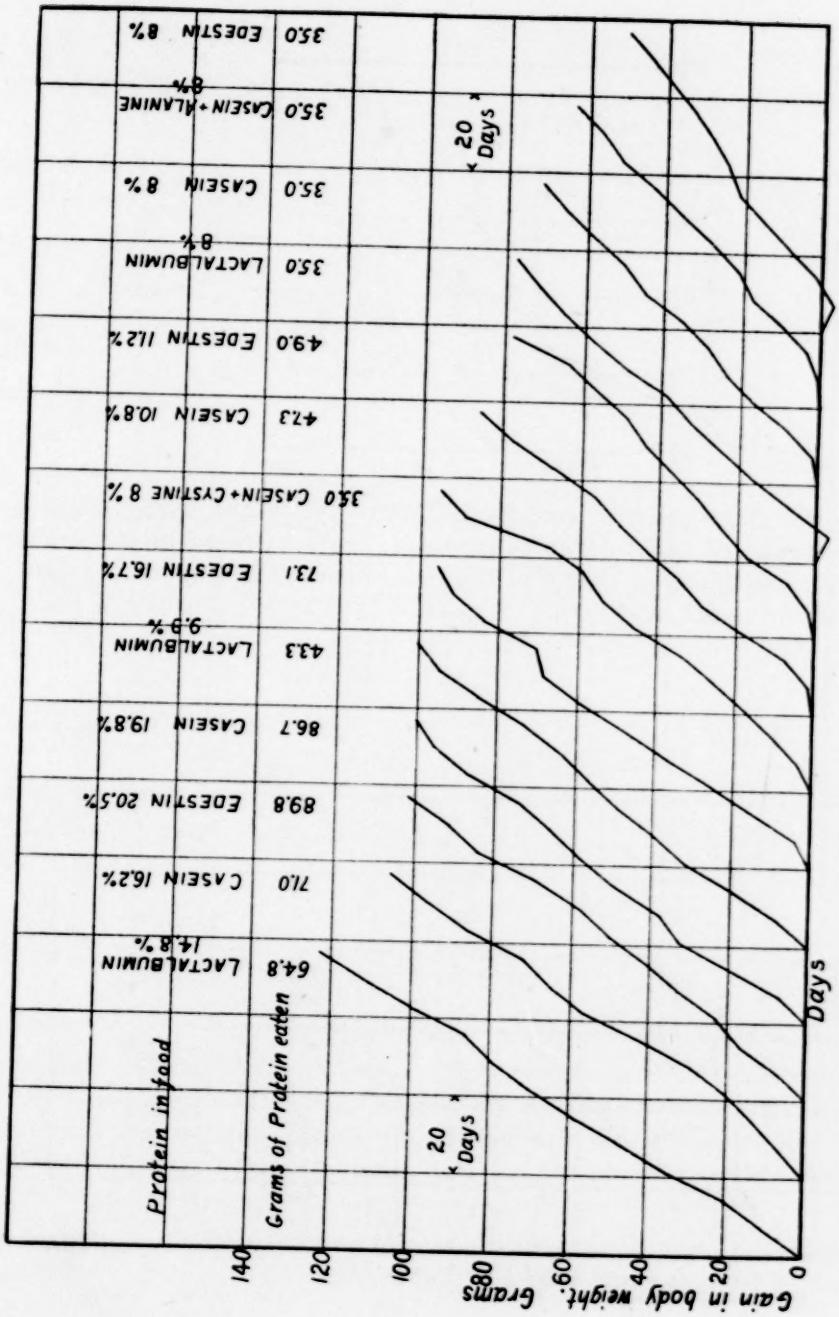

CHART I. Comparative growth indicated by the gains in weight of rats on the same intake of food containing different proteins in varying concentrations. Each experiment lasted 77 days. The graphs represent the composite results summarized in Table II.

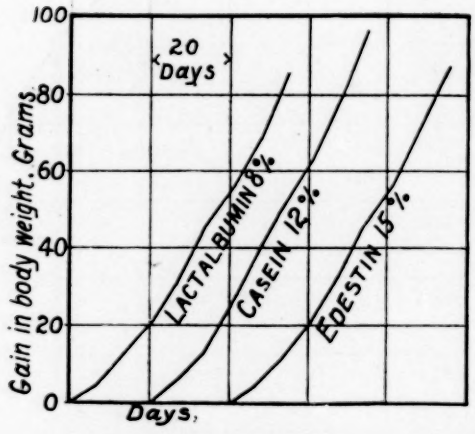

CHART II. Showing that comparable gains in weight were obtained by unlike intake of three different proteins, the total food intake being the same during the same length of time—56 days. The graphs represent the composite results summarized in Table IV.

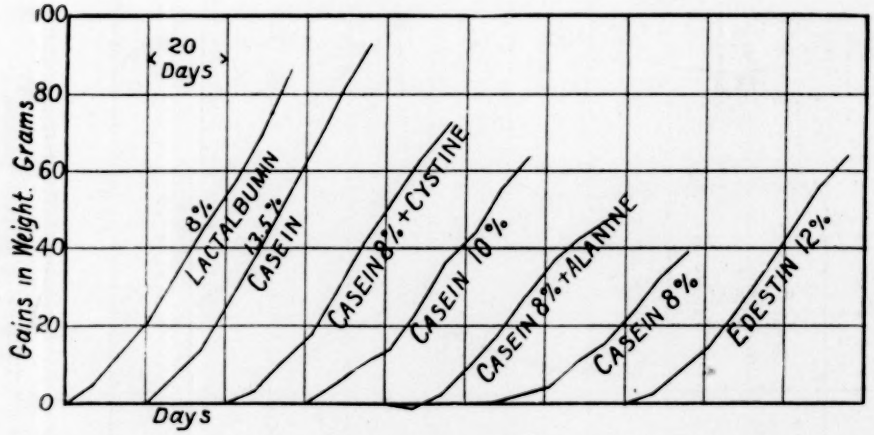

CHART III. Composite graphs of gains in weight under the comparable conditions of feeding summarized in Table IV.

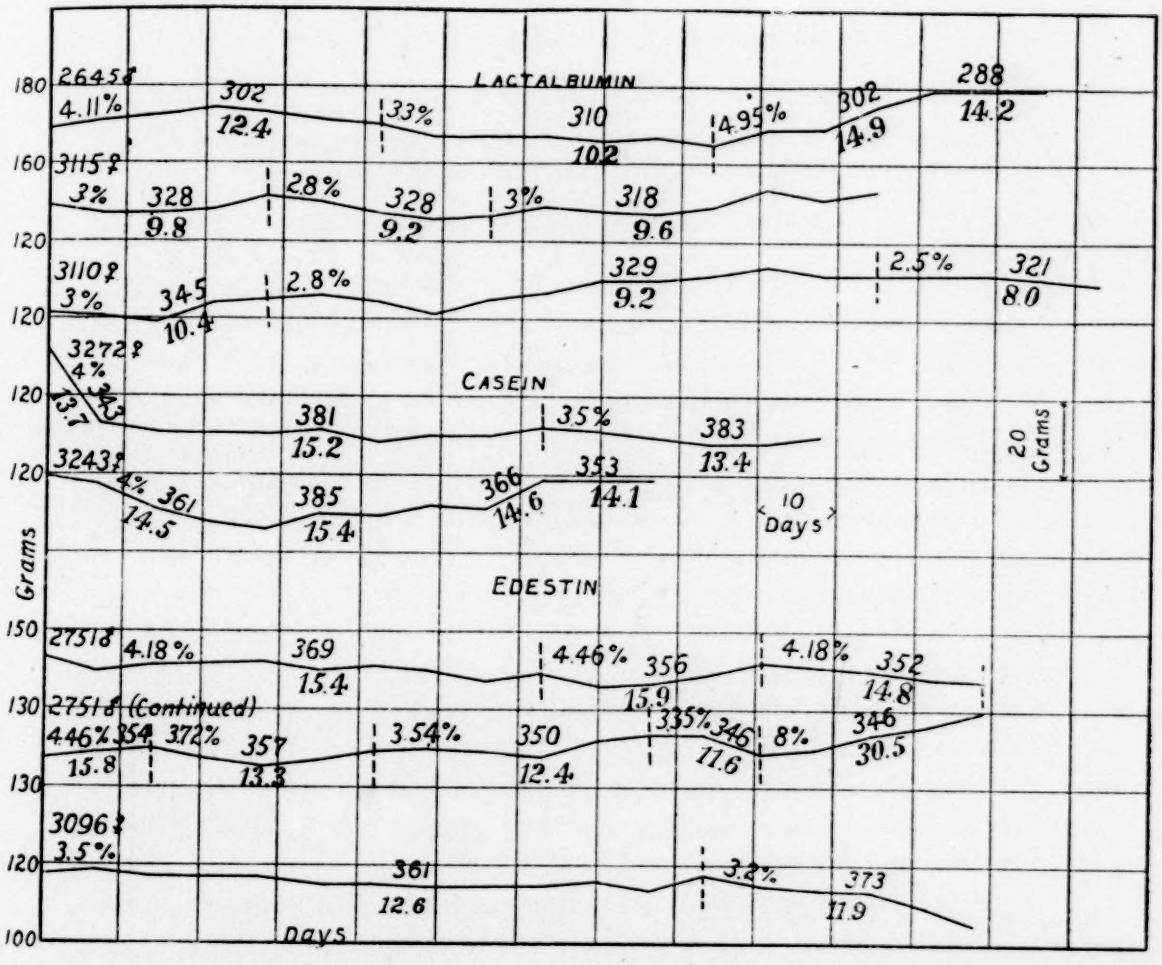

CHART IV. Illustrating changes of body weight involving successful or unsuccessful maintenance or increment of body weight on various absolute intakes of three different proteins. The nature and percentage of the protein added to the diet, together with the total food intake (above the line) and the protein intake (below the line) expressed in mg. per week per gm. of rat, are indicated on the curves.

THE EFFECT OF SURGICAL PROCEDURES ON BLOOD SUGAR AND RENAL PERMEABILITY.

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(Received for publication, June 10, 1916.)

By a study of the effect of surgical procedures on the blood sugar, Epstein and Aschner[1] have found that the blood sugar content after operations is invariably increased. The degree of elevation appears to be dependent principally upon the character and the duration of the anesthesia. From the extraordinary height to which the blood sugar rises after operation one would expect the presence of sugar in the post-operative urine to be of very frequent occurrence. However, glycosuria after operation occurs only occasionally, one case in fifty showing traces of sugar in the urine.[1]

These facts have led the above authors to conclude that the failure of sugar to appear in the urine might be ascribed to one or both of two causes: (1) that the hyperglycemia might be only "relative"—similar to that occurring after hemorrhage,[2] and (2) even if the observed hyperglycemia represented an actual increase in the amount of sugar in the blood, a glycosuria might not develop, because the kidneys might be rendered impermeable to sugar through the action of the anesthetic upon them.

The present study is supplementary to the one mentioned above. Its object is to ascertain to what extent renal function becomes affected by the operative procedures (anesthesia, etc.). In a recent paper by Epstein and Baehr[3] experiments are recorded which show that in the diabetes following pancreatectomy, im-

* Work done under the tenure of the Moses Heinemann Fellowship in Pathology.

[1] Epstein, A. A., and Aschner, P. W., *J. Biol. Chem.*, 1916, xxv, 151.

[2] Epstein, A. A., and Baehr, G., *J. Biol. Chem.*, 1914, xviii, 21.

[3] Epstein and Baehr, *J. Biol. Chem.*, 1916, xxiv, 1.

pairment of renal function leads to reduction or disappearance of the sugar in the urine with a consequent accumulation of sugar in the blood. This is in keeping with the observations on human beings recorded by Von Noorden,[4] Lépine,[5] and more recently by Epstein and Felsen.[6]

So that if the effect of the anesthesia and the other surgical procedures upon the kidneys is such that their function becomes impaired, it is more than likely that the failure of sugar to appear in the urine may be ascribed, at least in part, to a lessened renal permeability.

Method of Procedure.

The function of the kidneys was tested by means of phenolsulfonephthalein.

The cases selected for the study were those of simple surgical conditions in which no evidence of renal disease could be found. 24 hours prior to the operation the patients received 6 mg. of phenolsulfonephthalein intramuscularly, and the time of appearance, together with the amount of the dye excreted in 1 and 2 hours, was determined. On the day of the operation the blood sugar was determined directly before the anesthesia, and again after the operation. Another dose of phenolsulfonephthalein was also injected, and its elimination in the urine observed. The blood sugar was estimated by means of the microchemical method of one of the authors.[7] The phenolsulfonephthalein excretion was determined in the Hellige apparatus.

One of the effects of the surgical procedures common to all the cases studied was a marked diminution in the amount of urine secreted. This is undoubtedly due in part to the withholding of food from the patient prior to the operation, and to the loss of fluid from the body by sweating during the operation. Postoperative vomiting may also contribute to the loss of fluid.

The results obtained in the estimation of the blood sugar are in confirmation of those found in the work mentioned above.[1] A

[4] Von Noorden, C., Handb. Pathologie des Stoffwechsels, Berlin, 2nd edition, 1906, ii, 3.

[5] Lépine, R., *Rev. méd.*, 1897, xvii, 832.

[6] Epstein and Felsen, to be published.

[7] Epstein, A. A., *J. Am. Med. Assn.*, 1914, lxiii, 1667

Case.	Phenolsulfonephthalein excretion 24 hrs. before operation.				Blood sugar.		Phenolsulfonephthalein excretion directly after operation.				Urine for 24 hrs. after operation.		Operation.	
	Appearance.	1st hr.	2nd hr.	Total.	Directly before anesthesia	Directly after operation.	Appearance.	1st hr.	2nd hr.	Total.	Sugar.	Acetone.	Kind.	Duration.
	min.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*min.*	*per cent*	*per cent*	*per cent*				*min.*
C. P.....	13	37	17	54	0.110	0.172	60			26	0	+	Herniotomy.	54
I. B.....	15			78	0.104	0.154	25	41	20	61	0	+	Appendectomy.	13
M. B.....	45	35	14	49	0.142	0.174	20	15	14	29	0	+	Oophorectomy.	22
B. R.....	40	34	18	52	0.128	0.204	35	27	17	44	0	+	Radical breast amputation.	23
M. F.....	14	37	27	64	0.128	0.176	20	17	5	22	0	+	Appendectomy.	24
R. J.....	19	46	18	64	0.112	0.248	23	16	4	20	0	+	Radical breast amputation.	92
D. N.....	14	28	15	43	0.108	0.220	15	23	16	39	0	0	Tumor of colon.	14
V. B.....	15	32	18	50	0.168	0.224	25	5	33	38	0	0	Fistula in ano.	8
G. K.....	45	36	22	58	0.106	0.204	40	21	18	39	0	+	Ventral hernioplasty.	63
M. C.....	20	50	26	76	0.104	0.164	24	10	27	37	0	Trace.	Appendectomy.	17
M. B.....	12	24	20	44	0.124	0.220	30	10	2	12	0	"	Cholecystectomy.	52
A. N	14	17	15	32	0.184	0.272	30	8	20	28	0	"	Prolapse, rectal mucosa.	20

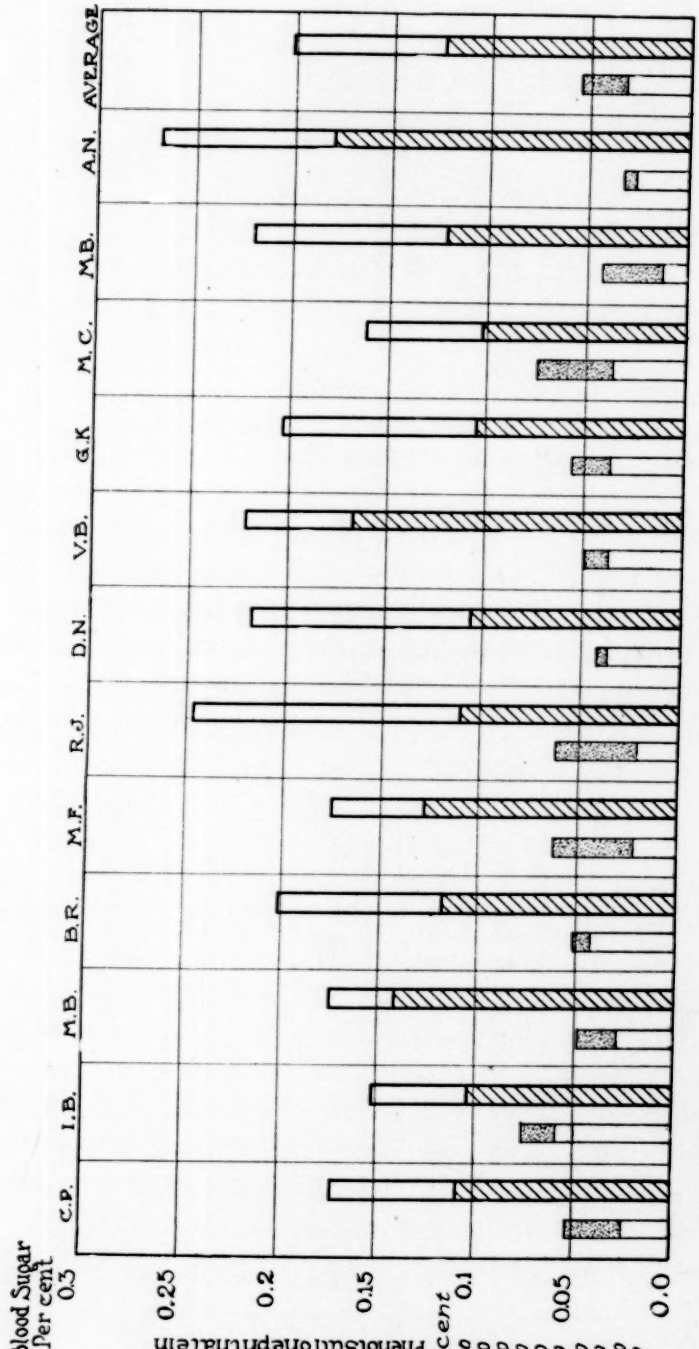

FIG. 1. Showing the post-operative rise in the blood sugar and the decrease in the phenolsulfonephthalein excretion. The dotted blocks represent the phenolsulfonephthalein, the cross-lined ones the blood sugar. The shaded portion of the dotted blocks represents the difference in the excretion of phenolsulfonephthalein before and after operation. The clear area in the cross-lined blocks represents the difference in the blood sugar.

comparison of the results obtained with phenolsulfonephthalein before and after operation shows a uniform delay in the time of appearance of the dye and a decrease in the amount excreted. The reduction in the phenolsulfonephthalein elimination is very striking in some of the cases (see table and Fig. 1). The decrease in the amount of phenolsulfonephthalein eliminated after operation as compared with that prior to operation ranges from 12 to 73 per cent, the average being about 25 per cent.

SUMMARY.

Operative procedures under anesthesia cause an increase in the blood sugar content (hyperglycemia), associated with a reduction or impairment of renal function. From this it is concluded that diminished permeability of the kidneys is responsible for the infrequent elimination of sugar in the urine after operations.

A COMPARATIVE STUDY OF THE BEHAVIOR OF PURIFIED PROTEINS TOWARDS PROTEOLYTIC ENZYMES.*

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(Received for publication, June 19, 1916.)

The present day students of protein metabolism are practically in agreement in the view that a protein must be disintegrated into its component amino-acids before it can be utilized by the animal body.[1] From this standpoint the importance of the digestive process becomes paramount and its study with the newer methods correspondingly necessary. In fact, the problem of protein utilization becomes in essence a problem of the digestibility of this foodstuff by the ferments of the gastro-intestinal canal.

For the further elucidation of the phenomena of digestion two methods of research are at present available. One, the investigation of the effect of proteoclastic enzyme preparations under suitable condition *in vitro;* the other, the study of the decomposition of the protein at various stages in its passage through the alimentary canal, by the fistula methods of London and his coworkers (1910). The main advantage of the latter method lies in the fact that the conditions of the experiment simulate the natural state of affairs most closely. Yet the *in vitro* method admits of a better standardization and control of the conditions and is therefore to be preferred in a comparative study.

A large number of studies of digestion *in vitro* have been carried out by investigators in the past, but for the most part the methods employed were inadequate for the solution of the problems of modern protein physiology.[2]

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* The data in this paper are taken from the dissertation presented by the author for the degree of Doctor of Philosophy, Yale University, 1916.

[1] Underhill, F. P., The Physiology of the Amino Acids, 1915.

[2] The earlier literature has been summed up by R. H. Chittenden in his book, On Digestive Proteolysis, New Haven, 1895.

The first workers concerned themselves with the changes in the solubility of the proteins as a result of the enzymatic action. Later investigators, following Kühne, became interested in the formation of proteoses and peptones. A variety of reagents were introduced for the purpose of effecting separations of the various groups, notably tannic acid, phosphotungstic acid, ammonium sulfate, and zinc sulfate (Zunz, 1899). Taylor (1907) in reviewing these methods has pointed out their shortcomings.

Physicochemical Methods.—With the advent of the physicochemical method of approach new modes of studying proteolysis were introduced. Among these were the conductivity method of Sjöquist (1895) which was later developed by Oker-Blom (1902) and Bayliss (1904, 1907). The cryoscopic depression was also used by Oker-Blom (1902). Changes in the hydroxyl ion concentrations of digestion mixtures have been used to measure proteolysis, both the gas chain and colorimetric procedures being employed. A recent development along these lines is the work of Christiansen (1912) who pointed out that the diminution of free acidity to Günzurg's reagent was an accurate measure of the extent of pepsin-hydrochloric acid digestion.

Optical Methods.—Schütz (1885) showed that changes occurred in the optical activity of digestion mixtures when proteolysis took place. Following him a number of investigators, notably Fischer and Abderhalden and their coworkers, have employed the polarimetric method The most recent application of this is the work of Abderhalden on the detection of proteolytic ferments in the blood. Other optical procedures have been used. Robertson (1912) attempted the use of the refractometer, but without success. Hirsch (1914) showed that the interferometer could replace the polariscope. Recently Kober and Haw (1916) have brought the use of the spectrophotometer into prominence in this connection.

Colorimetric Methods.—The intensity of the biuret reaction was first suggested by Liebermann as a means of measuring the extent of proteolysis. Vernon (1903) seems to have been the first to develop this procedure successfully as a quantitative method. The most modern aspect of this method is the work of Kober and Haw (1916) who claim that by the study of the extinction coefficients at various wave lengths they can measure spectrophotometrically the quantities of the amino-acids, di-, tri-, and tetrapeptides present in a given mixture.

Triketohydrindin hydrate (ninhydrin), first brought into prominence by the work of Abderhalden (1913), has since been applied for the colorimetric estimation of α-amino-acids (Herzfeld, 1914). Harding and MacLean (1915, 1916) improved upon Herzfeld's original method and have made it more generally available for the study of proteolysis.

A number of purely chemical procedures for following the digestion of proteins have been proposed. These are of two types, and since only those which actually deal with the estimation of amino groups meet the requirements of modern studies of proteolysis, the others, such as the carbamino quotient of Siegfried and Neumann (1908) and the bromine absorption methods of Brown and Millar (1906), Auld and Mosscrop (1913), and Siegfried and Reppin (1915), may be dismissed without further comment.

If we accept the concept of the structure of the protein molecule as laid down by Hofmeister and Fischer it follows that the best index of the extent to which a protein has been disintegrated is the ratio of the amino nitrogen at a given time to the total amino nitrogen obtained after complete hydrolysis. This ratio will be applicable to all proteins and is therefore well adapted for comparative work.

There are at present four methods by which the amino nitrogen of proteins and their derivatives may be estimated. The first chronologically is the "formol" titration of Sörensen (1908). This method has had a wide use; and in the study of proteolysis the most notable application has been in the investigation of Henriques and Gjalbaek (1911) who followed the cleavage of a number of proteins acted upon by acid, alkali, pepsin, and trypsin. Their experiments were carried out over long periods of time (108 days in some trials), and in the case of the enzyme experiments with a rather low ferment concentration. The data are, however, open to criticism for two reasons: their results are calculated on the basis of the total nitrogen of the proteins; and second, no account was taken of the quantities of amino nitrogen introduced with the ferments.

Van Slyke (1911, 1912, 1913) developed the nitrous acid method of estimating amino nitrogen so that it is now available for the study of digestion even where only small amounts of material are at hand. There has been some debate regarding the reliability of the method as applied to the products of proteolysis (Rice, 1915, Andersen, 1915). Abderhalden defends the accuracy of the method; and in the experience of the author the procedure has given very satisfactory results. The nitrous acid method has already found application in the proteolysis studies of Van Slyke (1911), White and Crozier (1911), White and Thomas (1912), Abderhalden and Pettibone (1912), and Berg (1916).

Attention should be called to the method proposed by Kober and Sugiura (1913). These authors find that it is possible to estimate amino nitrogen in protein derivatives on the basis of the fact that the α- and β-amino-acids dissolve a definite quantity of copper when treated with freshly precipitated copper hydroxide, and that the estimation of the copper in the complex formed is a convenient way of estimating amino nitrogen. They also point out that by an extension of their method the amino nitrogen in free amino-acids and in polypeptide linkage may be differentiated, since the copper complexes of the former are precipitated by barium hydroxide while those of the latter are not. The value of the procedure remains to be demonstrated.

The colorimetric method of Harding and MacLean (1915, 1916) of estimating α-amino nitrogen has been referred to above.

EXPERIMENTAL PART.

From the foregoing review it is apparent that for exact comparative work those procedures involving the estimation of amino nitrogen are most suitable. Of these it seemed advisable to use

the micro method of Van Slyke (1913) which has the additional advantage that it can be applied to small amounts of material. It may be argued that the methods of Kober and Sugiura and of Harding and MacLean are also applicable to small quantities; but the lack of experience with these methods made it appear as though the Van Slyke procedure, tested quite rigorously by many investigators, was on the whole best suited to the problem in hand. Since this work was begun, Harding and MacLean (1916) have published a preliminary report of digestion studies in which they demonstrated the applicability of their colorimetric method to proteolysis work.

Materials Used.—The *proteins* used in this study were prepared at the laboratory of the Connecticut Agricultural Experiment Station.[3] The *pepsin* used was a commercial scale preparation having considerable activity. The author is indebted to Dr. I. F. Harris for the sample of *trypsin* used. The *erepsin* employed was prepared as directed by Rice (1915) from the intestinal mucosa of the pig. This procedure yields an active solution of erepsin provided care is taken to scrape the mucosa and to place the scrapings under an antiseptic within a few hours after the animal has been slaughtered. It was found that 4 days autolysis followed by 4 days of dialysis, instead of 10 days for each process, called for by Rice, gave an active preparation which contained only very small quantities of amino nitrogen.

In gastric proteolysis at least two distinct factors, the pepsin and the hydrochloric acid, are involved. It might be supposed that the acid would act upon protein, causing it to be cleaved into simpler fragments with the liberation of free amino groups. Just how much of the digestion with pepsin and hydrochloric acid is due to the acid independently of the pepsin is as yet unknown. With this in mind two experiments were carried out in which the amounts of amino nitrogen liberated by pepsin and hydrochloric acid and the same concentration of hydrochloric acid alone under comparable conditions were determined.

A. Two portions of 50 cc. of diluted *egg-white* containing about 1 gm. of protein were mixed with an equal volume of 0.4 per cent hydrochloric acid. To one portion 20 mg. of pepsin were added. Both solutions were incubated in stoppered flasks at 38–40°C. and the amino nitrogen was estimated at intervals.

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[3] The author desires to express his thanks to Dr. Thomas B. Osborne for his generosity in supplying these purified preparations.

Proteolysis of Egg-White.

Hrs.	Amino nitrogen.	
	Pepsin + HCl.	HCl alone.
	mg.	*mg.*
0	5.1	5.1
6	12.2	
29	16.7	6.0
70	17.0	
92	20.3	5.4
122	20.8	

B. Two portions of 2 gm. each of *edestin* were treated with 100 cc. of 0.2 per cent hydrochloric acid. To one portion 20 mg. of pepsin were added. Both solutions were incubated in stoppered flasks at 38–40°C. and the amino nitrogen was determined at definite intervals.

Proteolysis of Edestin.

Days.	Amino nitrogen.	
	Pepsin + HCl.	HCl alone.
	mg.	*mg.*
0.12	19.4	5.5
0.37	24.5	
1	28.6	7.3
2	30.1	
10	47.0	9.4
58		19.0

The above tables show distinctly that *hydrochloric acid alone in the concentrations used has very little proteolytic effect and that the cleavage taking place in peptic digestion is due primarily to the catalytic effect of the pepsin added.*

Preliminary experiments indicated that simple suspension of the protein in the digestive fluid does not give satisfactory results. This is undoubtedly due, for the most part, to the difference in the surface of the protein exposed to the ferment action. Further, there was considerable difficulty in withdrawing a sample that could be regarded as uniform and at the same time leave the concentration of the solution with respect to protein unaltered. The following experiments were carried out to determine the extent of the differences that result in two parallel experiments due to inequalities in the surfaces exposed.

A. Peptic Digestion of Edestin.—In this case the protein went into solution almost immediately in both experiments so that the maximum surface was exposed to digestive action in both cases.

Two portions of 5 gm. of *edestin* were treated with 250 cc of 0.2 per cent hydrochloric acid containing 100 mg. of pepsin and incubated in stoppered flasks at 38–40°C. Samples of 25 or 30 cc. were taken at definite intervals, neutralized with solid magnesium oxide, and treated with 5 cc. of 5 per cent colloidal ferric hydroxide and 1 cc. of 50 per cent magnesium sulfate solution, and then diluted to 100 cc. The solution was filtered and an aliquot part, 60 or 70 cc., evaporated on the water bath and then washed into the measuring burette of the amino nitrogen apparatus. The results are calculated for the 5 gm. of protein used.

Proteolysis in Solutions.

Hrs.	Amino nitrogen.	
	I.	II.
	mg.	*mg.*
0	3.9	Lost.
4	34.2	34.2
11	46.0	47.4
19½	53.8	54.5
46	70.3	70.3

B. Two portions of 5 gm. of *edestin* were treated with 250 cc. of 0.4 per cent sodium bicarbonate solution containing 0.5 gm. of trypsin. Thymol was added as a preservative. The flasks containing the mixtures were stoppered and incubated, being shaken gently from time to time. Samples were taken at definite intervals as in the peptic digestions, and treated in the same way except that neutralization with magnesium oxide was omitted. Complete solution did *not* take place in the course of the experiment.

Proteolysis in Suspension.

Hrs.	Amino nitrogen.	
	I.	II.
	mg.	*mg.*
0	19.9	19.9
1	39.0	Lost.
3	63.0	65.7
6	86.0	90.5
11	124.0	136.5
19½	175.5	191.0
46	268.0	285.0

The data in the foregoing tables indicate clearly that *if accurate comparisons between proteins are to be made the digestions must be conducted on solutions of the proteins rather than suspensions.* The deviations may not always be as great as those obtained in Experiment B but it must be borne in mind that every precaution was taken here to have the digestions take place under strictly parallel conditions. Also with a purified protein in finely divided condition the chances for uniformity are much greater than in experiments like those of White and his coworkers (1911, 1912), Abderhalden and Pettibone (1912), and Berg (1916) where hashed tissues and finely chopped egg white were used as substrates.

Since it was not always possible to dissolve the proteins before proteolysis began it did not seem feasible, in the light of the above results, to attempt any comparative experiments with trypsin on native proteins. Instead, advantage was taken of the fact that under suitable conditions most proteins may be digested into solution with pepsin and hydrochloric acid in 3 to 6 hours so that the surface factor could be eliminated except for the slight variations that might occur in the early hours of peptic digestion. Since peptic digestion does not result in a very rapid cleavage of the protein molecule the divergences in the amounts of amino nitrogen liberated in the early hours of digestion before complete solution takes place are practically negligible. The plan of the experiments in which a large series of proteins were studied was as follows: The proteins were suspended in 0.2 per cent hydrochloric acid containing pepsin and then placed in an incubator at 38–40°C. The flasks containing the suspensions were gently agitated from time to time and when complete solution had taken place in all the flasks the sampling was begun. After the peptic digestions had run for several days and the increases from day to day were not very great, a portion of the solution was made alkaline with a slight excess of sodium carbonate and treated with trypsin. The action of the trypsin was continued for a week and then a solution of erepsin added. The amino nitrogen was estimated at intervals.

This method, in which all of the reacting material is in solution, gives the best possible conditions for adequate comparisons of the digestibility of different proteins. It is believed that the results also give a more accurate picture of the normal digestive process

in vitro than does any other type of *in vitro* experiment. The analytical data are probably accurate to within 2 or 3 per cent of the total amount estimated; so that variations of a greater magnitude among the different proteins may be regarded as significant since all the data have been calculated on a uniform basis.

Series A.—5 gm. of each of the proteins used were treated with 250 cc. of 0.2 per cent hydrochloric acid containing 0.1 gm. of pepsin and incubated in stoppered flasks at 38–40°C. At the end of 3 hours complete solution of the proteins had taken place and sampling was begun. At definite intervals 5 cc. of solution were withdrawn and treated with 0.5 cc. of N NaOH, to inhibit further peptic digestion, and then analyzed for amino nitrogen by the micro method of Van Slyke. A control experiment was run with all the reagents and ferments in the same quantities except that no protein was added. This was sampled and analyzed in the same way as were the protein digests, and the amounts of amino nitrogen found were deducted from the corresponding figures obtained on the protein solutions, so that the data recorded in the following tables are corrected for all extraneous matter that might yield nitrogen under the conditions of the analysis. After 96 hours of peptic digestion a portion of 200 cc. was withdrawn, made faintly alkaline with 10 cc. of 15 per cent sodium carbonate, and treated with 25 cc. of a trypsin solution equivalent to 0.4 gm. of dry trypsin. Tricresol was used as a preservative. The solutions were again incubated at 38–40°C. Samples were withdrawn at the stated times, treated with one-tenth their volume of glacial acetic acid to inhibit further proteolysis, and then analyzed for amino nitrogen. After a week 100 cc. portions of the tryptic digest were withdrawn and treated with 25 cc. of an erepsin solution and again incubated. Samples were taken and treated as in the case of the tryptic digest. The control experiment referred to above was carried out in the same way for the tryptic and ereptic digestions. The peptic, tryptic, and ereptic proteolyses were allowed to proceed until the experiment was stopped. The results in all cases were calculated for the 5 gm. of protein taken originally. The total amino nitrogen of each of the proteins was determined by hydrolyzing a definite amount of the substance with thirty or forty times its weight of 3 N hydrochloric acid after the method of Henriques and Gjalbaek (1910) by boiling in an autoclave at 140–150°C. for 2 hours. The hydrolysis solutions were then evaporated to dryness on a water bath to remove the excess of hydrochloric acid, and the residue was dissolved in a definite quantity of water and analyzed for amino nitrogen by the Van Slyke method. The amino nitrogen obtained on total hydrolysis was taken as the total amino nitrogen available. The data recorded in the columns "Per cent of total NH_2 N" were obtained by calculating the ratio of the amounts of amino nitrogen liberated at the various stages of digestion to the total available amino nitrogen.

Series B.—This was run under conditions quite comparable with those

in Series A except that the samples for analysis were taken at different intervals. The legumelin used in this series was from vetch. The three preparations of phaseolin employed were made in different ways. NaCl indicates that the globulin was prepared in the ordinary way by extraction with salt solution and precipitated by dilution. The NaOH preparation was obtained by extraction with diluted alkali and precipitation with acid. The NaCl-NaOH sample was obtained by treatment of the NaCl preparation with alkali and acid, as in the case of the NaOH material. These different preparations were studied with a view to explaining some of the differences observed by Osborne and Mendel[4] in feeding rats these materials as the sole protein of the dietary.

SERIES A.

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.
Casein. Total NH_2 N = 9.72 per cent.								
	mg.			*mg.*			*mg.*	
3	50	10						
9	64	13						
23	61	13						
47	67	14						
76	76	16						
94	69	14						
96			0	78	16			
98			2	193	40			
102			6	223	46			
108			12	245	50			
118.5			22.5	280	57			
144			48	301	62			
155	82	17						
168			72	312	64			
195			99	322	66			
239	Samples lost.							
263			167	332	68			
265						0	330	68
267						2	346	71
274						9	381	78
289						26	422	87
313						50	426	88
364						101	448	92
366			270	334	69			
370								

[4] Personal communication.

SERIES A—*Continued.*

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.
Cotton seed globulin. Total NH_2 N = 10.6 per cent.								
	mg.			*mg.*			*mg.*	
3	51	10						
9	62	12						
23	70	13						
47	77	14						
76	83	16						
94	85	16						
96			0	83	16			
98			2	217	41			
102			6	247	46			
108			12	281	53			
118.5			22.5	307	58			
144			4[illegible]	362	68			
155	94	18						
168			72	358	68			
195			99	384	72			
239	100	19						
263			167	389	73			
265						0	389	73
267						2	397	75
274						9	427	81
289						26	448	85
313						50	460	87
364						101	463	87
366			270	392	74			
370	121	23						
Edestin. Total NH_2 N = 11.02 per cent.								
3	48	9						
9	61	11						
23	71	13						
47	76	14						
76	82	15						
94	91	16						
96			0	93	17			
98			2	225	41			
102			6	259	47			
108			12	283	51			
118.5			22.5	309	56			
144			48	345	63			

SERIES A—*Continued.*

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.
	mg.			*mg.*			*mg.*	
155	90	16						
168			72	351	64			
195			99	356	65			
239	104	19						
263			167	378	68			
265						0	378	69
267						2	401	73
274						9	437	79
289						26	464	84
313						50	469	85
364						101	482	87
366			270	384	70			
370	117	21						

Gelatin. Total NH_2 N = 10.80 per cent.

3	23	4						
9	23	4						
23	25	5						
47	25	5						
76	27	5						
94	39	7						
96			0	47	9			
98			2	100	18			
102			6	131	24			
108			12	163	30			
118.5			22.5	191	35			
144			48	223	41			
155	41	8						
168			72	252	47			
195			99	265	49			
239	43	8						
263			167	299	55			
265						0	285	53
267						2	304	56
274						9	342	63
289						26	392	72
313						50	394	73
364						101	442	82
36			270	310	57			
370	51	9						

SERIES A—*Continued.*

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N
Coagulated lactalbumin. Total NH_2 N = 11.15 per cent.								
	mg.			*mg.*			*mg.*	
3	70	12.5						
9	80	14						
23	87	16						
47	89	16						
76	97	17						
94	110	20						
96			0	121	22			
98			2	229	41			
102			6	261	47			
108			12	296	53			
118.5			22.5	318	57			
144			48	366	65			
155	114	20						
168			72	371	66			
195			99	385	69			
239	127	23						
263			167	396	71			
265						0	390	70
267						2	404	72
274						9	429	77
289						26	466	83
313						50	460	82
364						101	489	88
366			270	394	71			
370	132	24						
Phaseolin (NaCl method). Total NH_2 N = 11.15 per cent.								
3	61	11						
9	73	13						
23	75	14						
47	91	16						
76	99	18						
94	103	19						
96			0	108	19			
98			2	173	31			
102			6	223	40			

SERIES A—*Continued.*

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N
	mg.			*mg.*			*mg.*	
108			12	257	46			
118.5			22.5	295	53			
144			48	338	61			
155	103	19						
168			72	370	66			
195			99	386	69			
239	109	20						
263			167	409	73			
265						0	420	75
267						2	432	77
274						9	436	78
289						26	500	90
313						50	484	87
364						101	510	91
366			270	428	77			
370	118	21						
Soy bean globulin. Total NH_2 N = 11.20 per cent.								
3	61	11						
9	70	13						
23	71	13						
47	83	15						
76	92	16						
94	100	18						
96			0	107	20			
98			2	187	33			
102			6	235	42			
108			12	274	49			
118.5			22.5	313	56			
144			48	353	63			
155	111	20						
168			72	374	67			
195			99	387	69			
239	121	22						
263			167	400	71			
265						0	391	69
267						2	411	73
274						9	428	76

SERIES A—*Concluded.*

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.
	mg.			*mg.*			*mg.*	
289						26	464	83
313						50	458	82
364						101	482	86
366			270	401	72			
370	136	24						
Squash seed globulin. Total NH_2 N = 11.06 per cent.								
3	61	11						
9	70	13						
23	79	14						
47	88	16						
76	91	16						
94	104	19						
96			0	100	18			
98			2	228	41			
102			6	278	50			
108			12	297	54			
118.5			22.5	332	60			
144			48	369	66			
155	106	19						
168			72	378	68			
195			99	394	71			
239	113	20						
263			167	413	75			
265						0	413	75
267						2	419	76
274						9	456	83
289						26	489	88
313						50	503	91
364						101	516	93
366			270	417	75			
370	120	22						

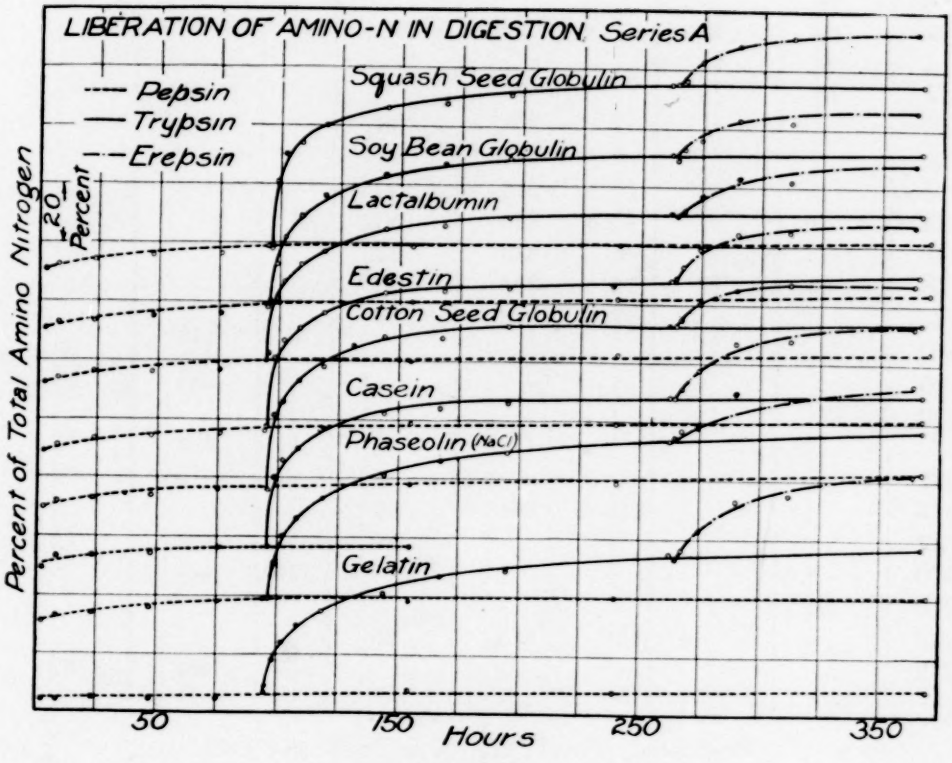
LIBERATION OF AMINO-N IN DIGESTION Series A
Pepsin
Trypsin
Erepsin
Squash Seed Globulin
Soy Bean Globulin
Lactalbumin
Edestin
Cotton Seed Globulin
Casein
Phaseolin (NaCl)
Gelatin
Percent of Total Amino Nitrogen
20 Percent
50
150
250
350
Hours

CHART A.

SERIES B.

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.
			Amandin. Total NH_2 N = 11.00 per cent.					
	mg.			*mg.*			*mg.*	
6	58	10						
21	63	11						
51	74	13						
94	80	5						
96			2	208	38			
100			6	255	46			
105.5			11.5	274	50			
114.5			20.5	310	56			
140			46	339	62			
168	96	18	74	374	68			
24			147	376	68			
260						0	377	69
265						5	384	70
270	101	18						
27						11	394	72
287						27	415	76
316			222	361	66			
317						57	402	74
370						110	415	76
416	105	19						
417			323	357	65			
419						159	452	82
			Conglutin. Total NH_2 N = 10.86 per cent.					
6	47	9						
21	61	11						
51	65	12						
94	67	12						
96			2	193	35			
100			6	229	42			
105.5			11.5	256	47			
114.5			20.5	276	51			
140			46	316	58			
168	83	15	74	338	62			
241			147	337	62			

SERIES B—*Continued.*

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.
	mg.			*mg.*			*mg.*	
260						0	342	63
265						5	347	64
270	91	17						
271						11	360	66
287						27	392	72
316			222	329	61			
317						57	382	71
370						110	398	73
416	97	18						
417			323	331	61			
419						159	463	85
Phaseolin (NaOH). Total NH_2 N = 10.72 per cent.								
6	68	13						
21	76	14						
51	83	15						
94	90	17						
96			2	155	29			
100			6	187	35			
105.5			11.5	212	39			
114.5			20.5	244	45			
140			46	296	55			
168	102	19	74	348	65			
241			147	372	69			
260						0	371	69
265						5	409	76
270	115	21						
271						11	394	74
287						27	420	78
316			222	374	70			
317						57	434	81
370						110	452	84
416	115	21						
417			323	372	69			
419						159	463	86

SERIES B—*Continued.*

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.
			Phaseolin (NaCl-NaOH). Total NH_2 N = 10.57 per cent.					
	mg.			*mg.*			*mg.*	
6	71	13						
21	78	15						
51	88	17						
94	94	18						
96			2	158	30			
100			6	207	39			
105.5			11.5	233	44			
114.5			20.5	254	48			
140			46	295	56			
168	114	22	74	351	66			
241			147	375	71			
260						0	393	74
265						5	390	74
270	126	24						
271						11	411	78
287						27	434	82
316			222	389	73			
317						57	427	81
370						110	469	89
416	117	23						
417			323	387	73			
419						159	481	91
			Phaseolin (NaCl). Total NH_2 N = 11.15 per cent.					
6	62	11						
21	79	14						
51	94	17						
94	94	17						
96			2	178	32			
100			6	220	39			
105.5			11.5	247	44			
114.5			20.5	276	49			
140			46	322	58			
168	108	20	74	362	65			
241			147	396	71			

SERIES B—*Concluded.*

Pepsin.			Trypsin.			Erepsin.		
Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.	Hrs.	NH_2 N.	Per cent of total NH_2 N.
	mg.			*mg.*			*mg.*	
260						0	404	72
265						5	408	73
270	111	20						
271						11	418	75
287						27	448	80
316			222	401	72			
317						57	432	77
370						110	482	86
416	109	20						
417			323	388	70			
419						159	514	92

Legumelin. Total NH_2 N = 10.86 per cent.

6	66	12						
21	80	15						
51	94	17						
94	107	20						
96			2	198	36			
100			6	246	45			
105.5			11.5	262	48			
114.5			20.5	295	54			
140			46	324	63			
168	110	20	74	367	68			
241			147	389	72			
260						0	400	74
265						5	402	74
270	123	23						
271						11	421	78
287						27	448	82
316			222	392	72			
317						57	436	80
370						110	489	90
416	123	23						
417			323	393	72			
419						159	492	90

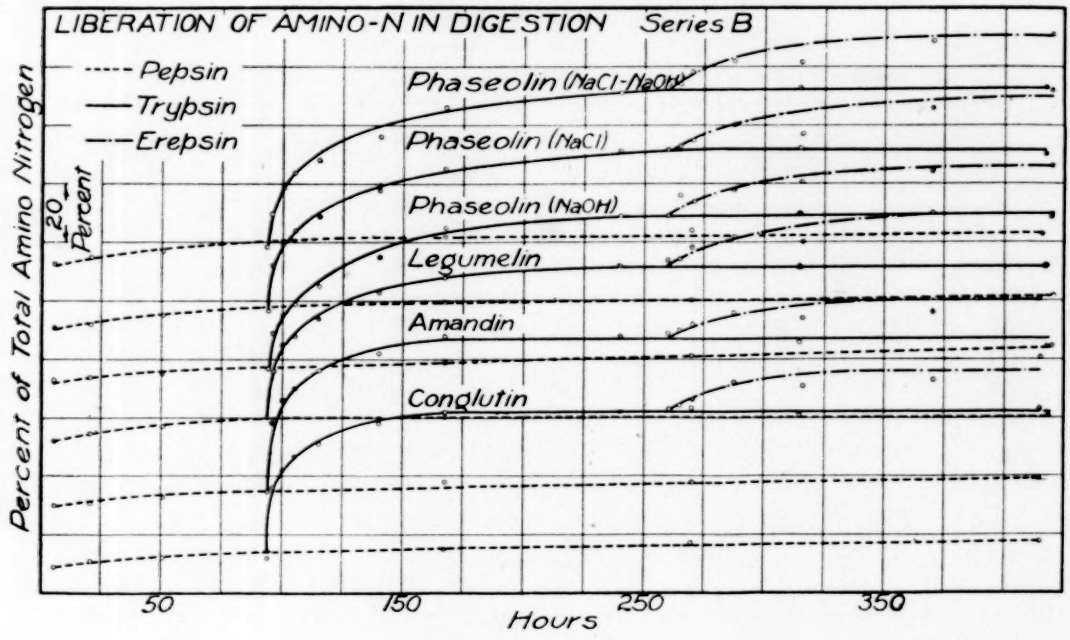

CHART B.

The data presented in the foregoing tables and curves indicate that there are no very striking differences in the extent of the disintegration, by the proteolytic ferments used, of the various proteins studied. Although the small variations noted between the amounts of amino nitrogen liberated from the different proteins at various stages of digestion may be of some significance physiologically, there seems to be no justification for this view at present. At least, there appears to be nothing in the data cited that could be used to explain the poor utilization of some of the leguminous proteins reported by Mendel and Fine (1912). While it is plausible that the defective utilization of these proteins may be due to their poor digestion *in vivo* this theory cannot be substantiated by these *in vitro* experiments. It may be that the periods during which the proteins were exposed to ferment action were too long to be comparable with the conditions that obtain *in vivo*. It has been shown that while native egg-white can be digested *in vitro* with little difficulty, when fed to a dog it is eliminated in the feces in a coagulable form (Bateman, 1916). In this case the protein leaves the stomach and intestine so rapidly that there is little or no time for the digestive ferments to act

upon it. Whether or not the same applies to the leguminous proteins is question for further experimentation to decide.

Viewed from the standpoint of hydrolytic cleavage the results presented are of some interest in that they show to what extent proteolysis may go on in a comparatively short time as a result of mild treatment. Previous investigators have claimed that it is possible to effect complete disintegration of proteins with enzymes. The periods of digestion, however, were quite long. Here we are able to present data which show that in comparatively short periods extensive hydrolysis is possible. Peptic activity liberates about 15 to 20 per cent of the available amino nitrogen in less than 100 hours. Trypsin acting on protein partially digested with pepsin effects a cleavage equivalent to about 70 per cent in 100 to 150 hours, while erepsin acting upon material that has been digested with pepsin and trypsin can disintegrate the protein so that only 10 to 15 per cent of the amino nitrogen is in peptide linkage. The results given above are perhaps of some interest in connection with the recent controversy between Abderhalden (1915) and Andersen (1915) regarding the possibility of getting complete digestion with ferments *in vitro*. It has been shown here that in some cases over 90 per cent of the total amino nitrogen may be liberated. It is even possible that the cleavage effected is greater than this because of the fact that certain errors are introduced in not making a correction for ammonia. Possibly we are not entirely justified in the assumption that the course of ammonia liberation runs parallel to that of the liberation of amino groups. It is quite true that the figure for amino nitrogen obtained on total hydrolysis is too high when no correction is made for the nitrogen of ammonium salts. If the amounts of ammonia liberated during digestion were low we should obtain a value for the ratio of the amino nitrogen liberated to the total amino nitrogen that is low when the correction for ammonia is not applied. The net result of such a condition would probably show that the extent of proteolytic breakdown is even greater than indicated by the figures above. In this respect the data presented above support the view of Abderhalden that it is possible to disintegrate protein almost completely by the action of ferments. A consideration of the ammonia data is now being undertaken with the hope of clearing up this point.

The curve of trypsin proteolysis makes it appear that the proteolytic activity of this enzyme is practically discontinued after 100 to 150 hours. This is in all probability due to the auto destruction of the ferment.

There are few data in the literature dealing with the behavior of trypsin towards purified proteins from a strictly quantitative standpoint. Van Slyke (1911) recorded one experiment on the digestion of edestin. Henriques and Gjalbaek (1911), using the "formol" titration, investigated the tryptic proteolysis of several pure proteins. Their experiments are not comparable with those presented in this paper since they were carried out over long periods of time with much smaller ferment concentrations than were used here; and they are further not strictly comparable among themselves, since no correction was made for the amino nitrogen of the ferment preparations added. It has further been pointed out above that the results are not calculated on the proper basis. It seemed desirable to determine how much difference in the curve of tryptic proteolysis was occasioned by a preliminary digestion of the protein with pepsin. Furthermore, the influence of successive additions of trypsin to proteolysis mixtures was studied with the view of establishing how far cleavage with trypsin alone may go. The following plan was carried out.

5 gm. of protein were dissolved in 225 cc. of 0.5 per cent sodium carbonate solution, and 25 cc. of a solution of trypsin in 0.5 per cent sodium carbonate were added.

An amount of dry trypsin equivalent to one-tenth of the protein used was taken, as in the previous experiments. Tricresol was used as a preservative and the solutions incubated in stoppered flasks at 38–40°C. Samples were withdrawn at stated intervals, treated with glacial acetic acid to inhibit further digestion, and then analyzed for amino nitrogen. A control experiment was run and adequate corrections were made for the ferment added so that the data recorded in the tables represent the amino nitrogen actually derived from the protein used. The results are calculated for the 5 gm. of protein employed and the cleavage is given as the ratio of the amino nitrogen liberated to the total available amino nitrogen.

After 5 days of digestion a 200 cc. portion of the solution was treated with 0.4 gm. of dry trypsin dissolved in 25 cc. of water, and the proteolysis followed as before. The results were calculated for the 5 gm. of protein originally taken and are comparable with those given elsewhere in this paper.

SERIES C.

Proteolysis with Trypsin.

Hrs.	Conglutin.		Phaseolin (NaOH).		Edestin.		Casein.		Phaseolin (NaCl).	
	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.
	mg.		*mg.*		*mg.*		*mg.*		*mg.*	
0	26	5	28	5	26	5	45	9	29	5
2	115	21	38	7	98	18	180	37	67	12
6	192	35	81	15	151	27	212	44	151	27
12	237	44	161	30	165	30	232	48	197	35
21	249	46	222	41	192	35	248	51	228	41
33	260	48	254	47	189	34	249	51	239	43
58	260	48	276	51	200	36	258	53	243	44
122	264	49	303	56	215	39	267	55	268	48
More trypsin added at the 122nd hr.										
127	292	54	302	60	237	43	272	56	294	53
144	311	57	345	64	281	51	287	59	309	55
168	310	57	349	65	302	55	297	61	321	58
222	314	58	359	67	322	58	319	66	330	59

A comparison of the data above with the results obtained when a preliminary peptic digestion was made indicates that the rate of digestion with trypsin is equally great in both cases. For example, in the case of casein the rates in the early hours are much the same in both types of digestion, the difference being found only when an examination of the later stages is made. It is shown that the same quantity of trypsin will eventually cause a much larger cleavage of the protein when a preliminary peptic digestion is made than is possible with the trypsin alone. This finding is in agreement with the observation of Fischer and Abderhalden (1903), who showed that neither proline nor phenylalanine could be obtained from the tryptic digestion of casein, while both of these amino-acids could be recovered if a preliminary peptic digestion preceded the action of trypsin.

The further addition of trypsin to a digestion mixture seems to be effective in causing further cleavage. Thus in the case of edestin the addition of more ferment caused an increase of 40 to 60 per cent in the amount of amino nitrogen liberated. The same

phenomenon was noted with the other proteins studied, though the increases were not quite so marked. The proteolysis effected by the initial quantity of trypsin is practically complete in less than 50 hours. This finding, coupled with the observation that additional trypsin can cause further degradation of the protein, appears to militate against the view of Abderhalden and Gigon (1907) regarding the inactivation of trypsin due to the binding of the enzyme by the end-products. If the end-products alone were responsible for the inactivation of the trypsin there should be no additional cleavage when more enzyme is added, for surely there must be a larger excess of end-products present over those necessary to bind the initial amount of trypsin. If the view of Abderhalden and Gigon were correct we should expect to find a definite end-point for tryptic proteolysis. Since this is not the case, as is evidenced by the results cited, it seems as though the inactivation of trypsin due to decomposition of the ferment is of greater moment than is the factor of combination with the end-products. The slight variations in dilution may presumably be ruled out because of the fact that the initial protein concentration is quite low (2 per cent). In general, where extensive cleavage is desired it would seem preferable to add small portions of ferment from time to time.

There are some data in the literature regarding the extent of proteolysis with pepsin and trypsin. However, as far as the author is aware, there are no quantitative studies of the extent to which pepsin followed by erepsin can disintegrate the protein molecule. It is generally believed that casein and gelatin are the only proteins attacked by erepsin.[5] In the case of other proteins preliminary cleavage by some other agency must be effected. The object of the experiments cited below was to determine the effect of erepsin upon protein that was partially digested with pepsin.

[5] On the basis of experiments with twelve proteins the author inclines to the view that erepsin may attack other native proteins. However, since the presence of trypsin in the sample of erepsin used could not be ruled out completely, though its presence was indeed doubtful (page 57), further experiments, intended to exclude the presence of trypsin, are planned. It is hoped that a report on this point will be ready soon.

3 gm. of protein were digested at 38–40°C., with 150 cc. of a 0.2 per cent solution of hydrochloric acid containing 60 mg. of pepsin. Proteolysis was allowed to proceed for 93 hours. A portion of 100 cc. equivalent to 2 gm. of protein was made alkaline with 10 cc. of 7.5 per cent sodium carbonate solution, treated with 25 cc. of erepsin solution, and incubated at 38–40°C. Samples were taken at intervals, treated with glacial acetic acid to stop further digestion, and then analyzed for amino nitrogen. The digestions were continued until no further increases of any considerable magnitude were noted. Series D and E were run under comparable conditions, but different ferment preparations were used. Control experiments were also run and corrections for the ferments introduced.

SERIES D.

Proteolysis with Pepsin and Erepsin.

Hrs.	Conglutin.		Soy bean globulin.		Edestin.		Casein.		Phaseolin (NaCl).	
	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.
	Peptic digestion.									
	mg.		*mg.*		*mg.*		*mg.*		*mg.*	
6	29	9	40	12	34	10	31	11	40	12
24	38	12	49	15	45	14	41	14	56	17
93	48	15	58	17	55	17	46	16	61	18
	Digests made alkaline and erepsin added at the 93rd hr.									
96	91	28	119	35	107	32	106	36	121	36
103	153	47	207	62	197	60	145	50	192	57
116	190	58	256	76	259	78	199	68	253	76
145	212	65	280	83	286	86	244	84	290	87
170	215	66	286	85	293	88	252	86	298	89
217	226	69	287	85	293	88	265	91	296	89

SERIES E.

Proteolysis with Pepsin and Erepsin.

Hrs.	Amandin.		Cottonseed globulin.		Coagulated lactalbumin.		Squash seed globulin.	
	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.	NH_2 N.	Per cent of total NH_2 N.
	Peptic digestion.							
	mg.		*mg.*		*mg.*		*mg.*	
6	34	10	39	12	47	14	40	12
25	47	14	49	15	59	18	49	15
93	49	15	52	16	66	20	61	18
	Digests made alkaline and erepsin added at the 93rd hr.							
96	67	20	71	22	89	27	72	22
103	116	35	120	38	140	42	120	36
117	180	55	175	55	194	58	171	51
145	252	76	245	71	240	72	242	73
173	275	83	258	81	258	77	284	86
212	272	83	262	82	277	83	289	87

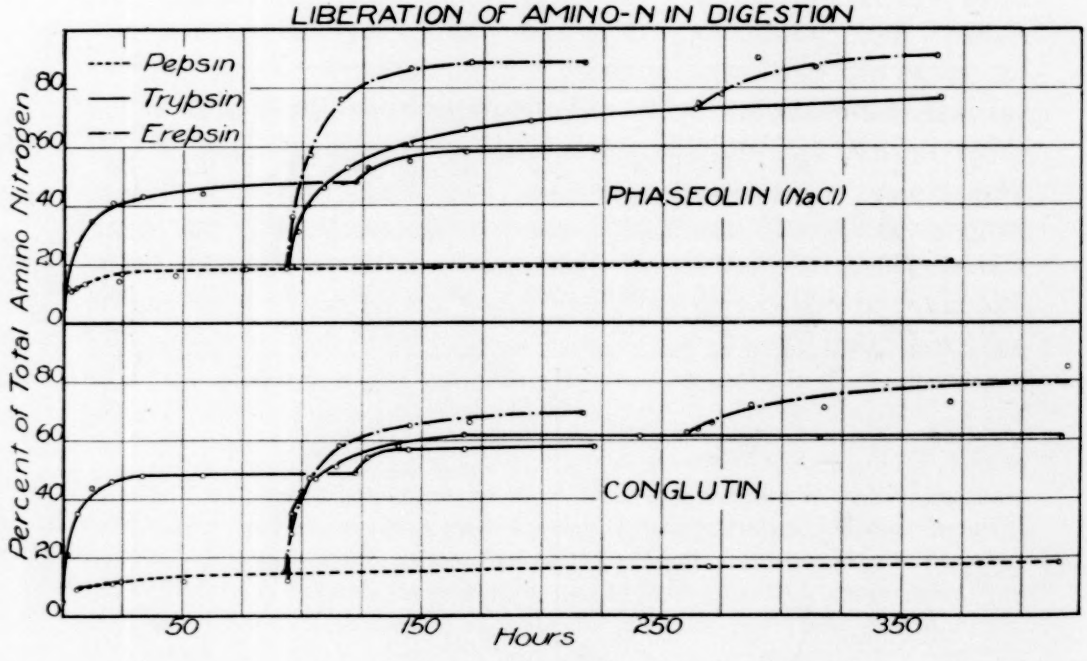

CHART C.

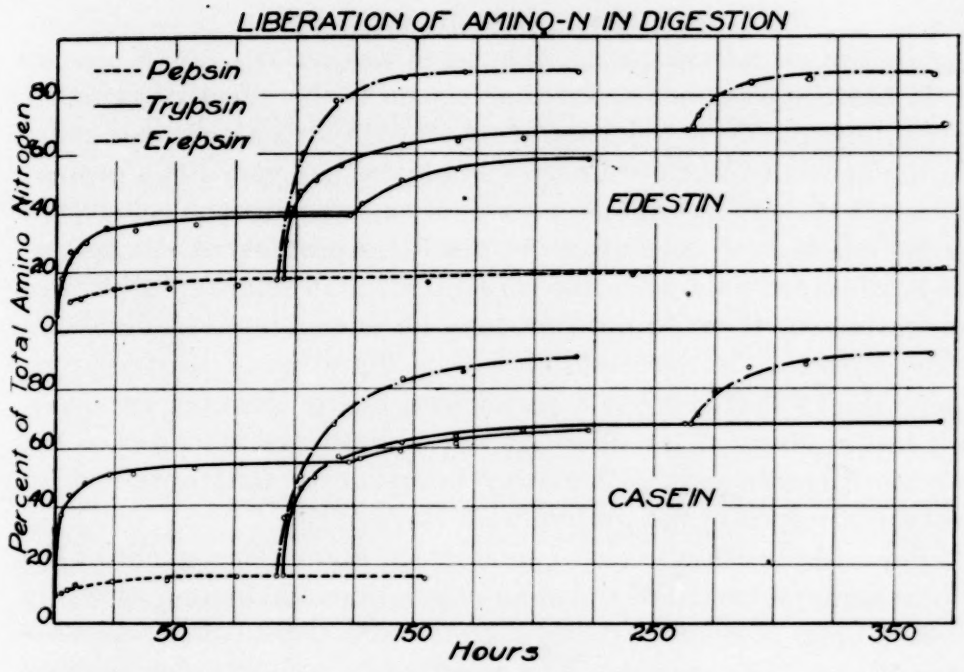

CHART D.

It will be noted from the tables and curves that erepsin can cause a far reaching cleavage of the protein molecule provided its action is preceded by a peptic digestion. Owing to the unlike ferment concentrations the rapidity of the cleavage varies somewhat in different preparations. The end results were, however, practically the same in the two series of digestions, the difference in action being noted only in the early hours of digestion.

Unfortunately no test was made for the presence of trypsin in the erepsin preparation used in Series D. However, the material employed in Series E, made in the same way, was tested with a flock of fibrin and no digestion was observed after incubation at 38° for 24 hours. Judging from the instability of trypsin in aqueous solution, especially on dialysis, the presence of trypsin in the erepsin solutions used (Rice's method) seems unlikely.

The desirability of having a means for effecting extensive proteolysis *in vitro* with mild treatment need not be emphasized. For such operations as the preparation of tryptophane or the isolation of other protein degradation products this method of rapidly disintegrating the protein molecule may prove of much service.

SUMMARY AND CONCLUSIONS.

It has been demonstrated that pepsin is the effective agent in pepsin-hydrochloric acid digestion since hydrochloric acid alone in the concentrations ordinarily employed has very little proteolytic effect. As a result of a series of experiments the conclusion seems justified that comparable results in proteolysis studies are to be obtained only when the substrates are in solution; otherwise deviations of 10 to 15 per cent may be encountered in duplicate experiments. An examination of the digestion of thirteen proteins shows that there is a parallelism in the cleavage curves of all the proteins, if the cleavage is calculated as the ratio of the amino nitrogen liberated at any one time to that obtained on total hydrolysis of the protein with strong acid.

Pepsin-hydrochloric acid can liberate about 20 per cent of the total amino nitrogen of a protein in less than 100 hours. Trypsin acting upon proteins partially digested with pepsin effects a cleavage of about 70 per cent. The action of trypsin upon native proteins can cause a cleavage of about 50 per cent of the peptide linkages. Further addition of trypsin may cause further disintegration of the protein.

Erepsin following the action of pepsin is a very effective agent in causing the disruption of the protein molecule. In two series of experiments cleavage of about 85 per cent of the protein could be demonstrated. The successive action of pepsin, trypsin, and erepsin liberates about 85 to 90 per cent of the total amino nitrogen of the protein studied.

The author wishes to express his thanks to Professor Lafayette B. Mendel, at whose suggestion this work was undertaken, for his advice and criticism.

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THE METABOLISM OF SULFUR.

I. THE RELATIVE ELIMINATIONS OF SULFUR AND NITROGEN IN THE DOG IN INANITION AND SUBSEQUENT FEEDING.

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(Received for publication, June 7, 1916.)

The suggestion has frequently been made that a study of the ratio of the sulfur to nitrogen excretion might give valuable evidence concerning the rate of metabolism of the protein molecule, and many experimental facts on this subject have accumulated in recent years.[1] Certain observers, notably von Wendt, maintain that no true picture of protein catabolism can be obtained from a study of the nitrogen excretion alone, but only from a study of both nitrogen and sulfur excretions. In general it has been observed that the maximal sulfur elimination precedes or coincides with the maximal nitrogen elimination. From these data it has been argued that the sulfur-rich moiety of the protein molecule is split and oxidized first by the organism, while the oxidation of the sulfur-poor fraction of the molecule is delayed. A large mass of experimental data concerning the course of the excretion of sulfur and its relation to nitrogen elimination in man in starvation is also available.[2] Of these studies, the most recent, the record of a

[1] Sivén, V. O., *Skand. Arch. Physiol.*, 1901, xi, 308. Sherman, H. C., and Hawk, P. B., *Am. J. Physiol.*, 1900–01, iv, 25. Von Wendt, G., *Skand. Arch. Physiol.*, 1905, xvii, 211. Ehrström, R., *ibid.*, 1906, xviii, 281. Falta, W., *Deutsch. Arch. klin. Med.*, 1905, lxxxvi, 517. Hämäläinen, J., and Helme, W., *Skand. Arch. Physiol*, 1906, xix, 182. Wolf, C. G. L., and Österberg, E., *Biochem. Z.*, 1912, xl, 193, 234; 1912, xli, 111. Cathcart, E. P., and Green, H. H., *Biochem. J.*, 1913, vii, 1. Tsuji, K., *Biochem. J.*, 1915, ix, 439.

[2] Compare Benedict, F. G., *Carnegie Institution of Washington, Publication No. 77*, 1907, 397, for a review of the older literature.

31 day fast reported by Benedict,[3] may be cited as typical. As the fast progressed there was a slight tendency for the ratio of the nitrogen to sulfur excretion to decrease; *i.e.*, the excretion of sulfur became relatively higher, although the ratios tended to remain within rather narrow limits, especially toward the end of the experiments. For the fasting dog data are less numerous and confined to short periods. In two experiments extending over a period of 7 days, reported by Underhill and Kleiner,[4] wide variations in the N:S ratio were observed although in one animal (Dog 4) the ratio was practically constant in the last 3 days of the fast (13.2, 13.5, and 13.6, calculated from the data of Underhill and Kleiner). Fasts of 3 to 4 days following ingestion of a high protein diet have been reported by Österberg and Wolf,[5] but because of the short periods and the previous high protein diet, these figures are best interpreted as showing relative lags in the elimination of nitrogen and sulfur after the superimposition of a high protein diet. Later observations by the same experimenters[6] included fasts of 8 days. As a result of these experiments Wolf and Österberg conclude that the organism protects the sulfur fraction of the protein molecule more effectively than the nitrogen, pointing to a special function of the cystine fraction of the protein molecule. It must be borne in mind, however, that the serum protein fed following the fasting period was higher in sulfur content than are the ordinary proteins of the diet[7] or the proteins of the body tissue with the exception of certain albuminoids (N:S = 10.9 for the protein fed, calculated from the data of Österberg and Wolf, as compared with 14 to 15 for ordinary muscle tissue). Ingestion of such a sulfur-rich protein following fasting would tend to result in lower N:S ratios than would be obtained with a more normal diet, and would cause the contrast between the normal and fasting ratios to appear more marked

[3] Benedict, *Carnegie Institution of Washington, Publication No. 203*, 1915, 277. A table comparing the results of this study with those obtained in previous studies is given on p. 279.

[4] Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 165.

[5] Österberg and Wolf, *Biochem. Z.*, 1907, v, 304.

[6] Wolf and Österberg, *Biochem. Z.*, 1911, xxxv, 329.

[7] Osborne, T. B., *Connecticut Agric. Exp. Station, Ann. Rep.*, 1900, 443. Compare also Sherman, H. C., *U. S. Dept. Agric., Bureau of Experiment Stations, Bull. 121*, 1902, 10.

than would have been the case had the diet been more nearly normal for such an animal. No sulfur determinations were reported by Howe, Mattill, and Hawk[8] in connection with a 117 day fast, the longest fast on record for the dog. No long continued fasting experiments on the dog in which the course of the sulfur elimination was followed and in which trustworthy methods for the determination of sulfur were used could be found in the literature.

McCollum[9] has suggested on the basis of feeding experiments with swine maintained on incomplete proteins that the processes of cellular catabolism and repair do not necessarily involve the destruction and resynthesis of the entire protein molecule. If such a partial degradation of the protein molecule occurs, it would seem probable that the sulfur-containing complex would be conserved by the organism, since the sulfur requirements of the body must be supplied by organic sulfur, and since cystine is considered an essential amino-acid which must be present preformed in the diet. The central nervous system contains considerable amounts of sulfur in the form of lipoids, the exact nature of which is not clearly defined. In the organism of the dog, the demand for sulfur is presumably greater than in man, because of the large amounts of sulfur lost through the shedding of the hair. However, the sulfur-rich protein in the dog does not form a part of the more essential tissues, as the heart, nervous system, etc., which are the last to suffer loss in fasting.[10] If the sulfur-containing complex can be retained for purposes of resynthesis, the withdrawal of protein (and cystine) from the diet as in fasting might result in a rise of the N : S ratio, indicating a conservation of sulfur, a rise which should become more marked as the fast progressed.

Inasmuch as there was found to be a paucity of data on the sulfur excretion by the fasting dog, such a study of a prolonged period of fasting followed by shorter periods of alternate feeding and fasting has been made, in the hope that it might prove of

[8] Howe, P. E., Mattill, H. A., and Hawk, P. B., *J. Biol. Chem.*, 1912, xi, 103.

[9] McCollum, E. V., *Am. J. Physiol.*, 1911–12, xxix, 215.

[10] Kumagawa, M., *Mittheil. med. Fakultät. kais. Japan. Univ., Tokio*, iii, No. 1, quoted by Lusk, G., The Elements of the Science of Nutrition, Philadelphia, 2nd edition, 1909, 76.

value from the point of view of a conservation of the sulfur of the protein molecule for resynthesis as discussed above. The subject of the experiments was a white female bull dog, which remained in excellent condition throughout the experiment. Water was available at all times. The animal was kept in a metabolism cage and the urine separated into 24 hour periods by catheterization. Analyses of the urine for total nitrogen by the method of Kjeldahl-Gunning and for total sulfur by the method of S. R. Benedict were made daily. In Table I for the sake of brevity the results are expressed in periods of 3 days for the most part, and the average daily elimination is given. Unfortunately no analyses were made during the first 4 days of the experiment. On the 40th day of the fast 600 gm. of beef heart[11] (N = 2.85 per cent, S = 0.191 per cent, N:S = 14.9) were fed, after which a second fasting period of 7 days followed. Beginning with the 48th day alternate feeding and fasting periods of various lengths were studied as detailed in the table.

No evidence of any increasing retention of sulfur as compared with nitrogen can be obtained from the experimental data. The N:S ratios for the first and last periods of the fast (Periods 5 to 7 and 37 to 39) are practically identical, 13.9 and 14.0, respectively. The lowest ratio, 12.9, was observed toward the end of the fasting experiment (Periods 34 to 36), and the highest, 16.1, toward the middle of the fast (Periods 15 to 17). With these two exceptions the ratios vary within narrow limits, from 14 to 15 for the most part. These ratios certainly show no tendency to rise higher than the normal feeding ratios. In fact, evidence of the opposite tendency was obtained. During Periods 67 to 73, in which 600 gm. of beef heart containing 17.1 gm. of nitrogen and 1.15 gm. of sulfur (N:S = 14.9) were fed daily, and in which a large part of the dietary nitrogen was excreted in the urine, indicating that the urgent need for protein for repair had been satisfied (compare Periods 40 and 48 in which the same amount of food was ingested), the ratios average 17.3. This ratio is higher than is the fasting ratio. Other experiments on dogs maintained

[11] For the determination of total sulfur in meat the sample was heated on the water bath with concentrated nitric acid, evaporated to dryness, and Benedict's method employed. Compare Wolf and Österberg, *Biochem. Z.*, 1910, xxix, 429.

TABLE I.

The Elimination of Nitrogen and Sulfur during Inanition and Subsequent Feeding. White Female Bull Dog.

Period.	Weight.	Total N for period.	Average daily N.	Total S for period.	Average daily S.	N : S	Remarks.
	kg.	*gm.*	*gm.*	*gm.*	*gm.*		
5– 7		8.59	2.86	0.595	0.198	13.9	Fasting.
9–11	15.78	7.67	2.56	0.517	0.172	14.8	"
12–14	15.29	6.99	2.33	0.498	0.166	14.0	"
15–17	14.82	5.94	1.98	0.370	0.123	16.1	"
18–20	14.42	6.32	2.11	0.458	0.153	13.8	"
21–24	14.00	8.09	2.03	0.581	0.145	14.0	"
25–27	13.63	6.13	2.04	0.446	0.149	13.6	"
28–30	13.26	6.22	2.07	0.416	0.139	14.8	"
31–33	12.84	6.09	2.03	0.414	0.138	14.7	"
34–36	12.27	6.06	2.02	0.468	0.156	12.9	"
37–39	11.97	5.47	1.82	0.390	0.130	14.0	"
40	12.14	10.22	10.22	0.526	0.526	19.44	600 gm. meat.
41	11.89	3.14	3.14	0.135	0.135	23.3	Fasting.
42–44	11.77	4.71	1.57	0.410	0.137	11.4	"
45–47	11.52	4.55	1.52	0.366	0.122	12.4	"
48	11.57	6.74	6.74	0.289	0.289	23.7	600 gm. meat.
49	11.39	2.59	2.59	0.201	0.201	12.9	Fasting.
50–51	11.48	12.02	6.01	0.730	0.365	16.5	300 gm. meat daily.
52–53	11.34	20.69	10.35	1.026	0.513	20.1	600 " " "
54*	12.02						600 " " "
55–56	12.11	21.25	10.63	1.178	0.589	18.0	600 " " "
57	12.12	12.70	12.70	0.734	0.734	17.2	600 " " "
58–60	11.81	8.44	2.81	0.683	0.228	12.3	Fasting.
61–63	11.37	7.03	2.34	0.540	0.180	13.0	"
64	10.90	2.29	2.29	0.138	0.138	16.7	"
65	11.01	13.00	13.00	0.484	0.484	26.9	600 gm. meat daily.
66	11.10	13.66	13.66	0.722	0.722	19.5	600 " " "
67	11.16	14.98	14.98	0.884	0.884	16.9	600 " " "
68–73	11.17	86.95	14.49	4.985	0.831	17.4	600 " " "
74	11.47	4.84	4.84	0.412	0.412	11.3	Fasting.
75	11.32	3.16	3.16	0.293	0.293	10.8	"
76–78	10.99	7.91	2.64	0.636	0.212	12.5	"

* Urine lost.

at various levels of protein intake but with the other foodstuffs also present in the diet, gave ratios averaging from 15 to 17. A protocol of such an experiment is given in Table II. If a partial

conservation of the protein molecule in cellular catabolism can be effected by the organism, as suggested by McCollum,[9] this physiological economy would not seem to be effective in the case of the sulfur fraction of the molecule, judging from the results of the present experiment. This conclusion is in accord with the present day conception of the indispensability of adequate amounts of preformed cystine in the diet, as recently exemplified by the experiments of Osborne and Mendel[12] on white rats. Results obtained in this laboratory[13] also indicate that dogs may be maintained in nitrogenous equilibrium on a low protein diet, poor in sulfur, with the addition of small amounts of cystine, although ingestion of the same diet without the addition of the cystine results in a negative balance.

TABLE II.

Dog A. Black Long-Haired Female.

Day.	Weight.	Total N.	Total S.	N : S	Diet.
	kg.	*gm.*	*gm.*		
1	17.20	4.44	0.202	21.9	180 gm. beef heart, 30 gm. lard, 70 gm. sucrose, 5 gm. $Ca_3(PO_4)_2$, water 400 cc. N = 5.13 gm., S = 0.344 gm.
2	16.95	4.46	0.265	16.8	
3	16.85	4.29	0.271	15.8	
4	16.96	3.92	0.217	18.0	
5	16.97	3.99	0.257	15.5	
6	16.97	4.22	0.274	15.4	
7	16.91	3.96	0.249	15.9	
Average..		4.18	0.248	16.9	
8	16.94	2.62	0.179	14.6	Beef heart 40 gm., sucrose 80 gm., starch 40 gm., lard 40 gm., $Ca_3(PO_4)_2$ 8 gm., water 400 cc. N = 1.14 gm., S = 0.076 gm.
9	16.93	2.14	0.152	14.1	
10	16.83	2.14	0.105	20.3	
11	16.73	2.04	0.150	13.6	
12	16.72	2.11	0.127	16.6	
13	16.65	1.85	0.115	16.0	
14	16.66	1.97	0.131	15.3	
Average..		2.12	0.137	15.4	

A brief consideration of the relative eliminations of nitrogen and sulfur on ingestion of a high protein diet subsequent to prolonged fasting serves to bring out certain points of interest. On

[12] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

[13] Unpublished data.

the 40th day 17.1 gm. of nitrogen and 1.15 gm. of sulfur were fed in the form of 600 gm. of beef heart. On this and the succeeding day (fasting) there is a marked rise in the N:S ratio, indicating a sulfur retention in the organism greater than the nitrogen retention. Calculating on the basis of the ratio of the preceding period, 14.0, the elimination of 13.36 gm. of nitrogen on these 2 days should have been accompanied by the elimination of 0.945 gm. of sulfur, instead of 0.661 gm. as observed. This may be interpreted as a retention of 0.293 gm. of sulfur above the amount which would be anticipated from the quantity of nitrogen retained. All the feeding periods following fasting show similar high ratios with a like retention of sulfur. When the feeding is continued over a number of days, there results a gradual diminution of the ratio till the normal ratio is reached (compare Periods 52 to 57 and 65 to 73). If, however, the feeding period be followed by a fasting period, the ratios are lower than the normal or fasting ratios, indicating an excessive elimination of sulfur as compared with nitrogen (Periods 42 to 47, 49, 58 to 64, and 74 to 78).

These facts are capable of interpretation in two ways. The retention of sulfur may be considered as due to a lag in sulfur elimination, and the increased elimination in fasting periods subsequent to high protein periods as due to a completion of the lag excretion. But it seems hardly probable that this lag would extend throughout a period of 7 days as in Periods 41 to 47. Moreover, data on the comparative rates of sulfur and nitrogen elimination in man and dogs under various conditions of diet[1] fail to afford evidence of so prolonged a lag of sulfur behind the nitrogen. The possibility that we are dealing with a specific demand for sulfur to build up sulfur-rich proteins, that is, with a definite retention for anabolic purposes, must also be considered. On the basis of this assumption, the gradual return to normal of the N:S ratio on continued feeding would represent the rebuilding of the sulfur-rich tissues, a restoration of material lost during the starvation period. The lower ratios of the fasting periods subsequent to feeding would indicate catabolism of this sulfur-rich protein formed on ingestion of food. Until more exact figures for the sulfur and nitrogen content of the various tissues and organs are available, further discussion of this point would be of little value. We believe, on the basis of unpublished experi-

ments already referred to[18] on the influence of feeding small amounts of cystine with a low protein diet, that this latter interpretation is correct and that we are dealing with a retention of sulfur in response to a specific demand by the organism and not with a lag in the excretion.

PHYSIOLOGY OF THE PHENOLS.*

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INTRODUCTION.

The work of Baumann, Brieger, Salkowski, and others has seemingly solved the problem of the origin of phenols in urine. It is clear that the urinary phenols arise from intestinal putrefaction, and that they are derived from the tyrosine portion of the protein molecule. However, with the exception of the work of Folin,[1] little has been done—due probably to the lack of a suitable method—to determine (1) the extent to which phenols may be formed under both normal and pathological conditions, and (2) the relationship between the free and conjugated phenols. It was with these objects in view that the present work was undertaken.

HISTORICAL.

The literature has to a certain extent been reviewed by Folin,[1] so that it will be necessary only to record the results of some recent investigations.

The elimination of phenols is increased by absorption from wounds and abscesses.[2]

The relative ability of the various organs to dispose of phenol is as follows: liver, kidney, muscle, brain, and blood; *i.e.*, the liver has the greatest capacity for conjugating phenols, while the blood has the least. Only the epithelia of the intestinal tract give results comparable to those obtained with the liver.[3] The conjugating function of the liver is not much impaired by disease.[3]

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* Dissertation for the degree of Ph.D., University of Pennsylvania, 1916.

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 309.

[2] Hammarsten, O., Physiological Chemistry, New York, 1911, 689.

[3] Herter, C. A., and Wakeman, A. J., *J. Exp. Med.*, 1899, iv, 307.

On starving, the phenols, after an initial fall, increase considerably.[4] Müller[5] reported the case of a man who showed an increase of 155 mg. of phenol on the 9th day of starvation. This was held to be due to putrefaction of intestinal secretions. If, while an animal is starving, phlorhizin be given, there is an increase in the output of phenols, but not until sugar makes its appearance.[6] It is the increased protein breakdown, rather than intestinal putrefaction, that accounts, under these circumstances, for the increase in the elimination of phenols. Lewin[6] observed in rabbits an increase from 6.91 mg. of phenol to 16.35 mg. of phenol per day, on giving 0.8 gm. of phlorhizin subcutaneously. In man there was an increase of two or three times the original amount of phenol upon administering 0.25 gm. of phlorhizin *per os* or subcutaneously.

It is well known that if a measured amount of phenol be ingested it cannot be recovered quantitatively in the urine, and it is believed that the missing fraction is burned. Tauber[7] fed phenol to a dog and found that as the dose was decreased, the amount of phenol oxidized increased. Thus, he found no phenol in the fore-period, either in the urine or in the feces. On feeding 0.24 gm. of phenol in water *per os*, he found 110 mg. of phenol in the urine, and only 9 mg. in the feces. In other words, about 53 per cent of the ingested phenol was oxidized in the body through oxalic acid to carbon dioxide. Heffter[8] states that after feeding phenol, there is an increase in phenolsulfuric acid but not in free phenol. On the other hand, Reale[9] reports that poisoning with large amounts of phenol results in the presence of free, as well as combined, phenol in the urine.

Jonescu[10] found that after feeding *p*-cresol to dogs, kept on a diet of horse meat, only about 25 per cent of the amount ingested is eliminated, and that the elimination is complete in 24 hours. Taking an average of nine persons, Siegfried and Zimmerman[11] showed that of the total phenols eliminated, 58.1 per cent is *p*-cresol, and 41.9 per cent is phenol. They[12] found also that *p*-cresol, fed to dogs, is largely changed to phenol. Thus, on feeding 0.5 gm. of *p*-cresol with 0.5 gm. of sodium bicarbonate on 4 successive days, 32 per cent of the total amount fed was recovered in the urine. Similarly, on feeding 0.8 gm. of *p*-cresol, 48 per cent was recovered.

The occurrence of the phenols in the urine upon disinfection of the intestine is shown in an experiment carried out by Baumann.[13] A dog

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[4] Herter, C. A., Chemical Pathology, Philadelphia, 1902, 425.

[5] Müller, F., *Berl. klin. Woch.*, 1887, xxiv, 405, 436.

[6] Lewin, C., *Beitr. chem. Phys. u. Path.*, 1902, i, 472.

[7] Tauber, E., *Z. physiol. Chem.*, 1878–79, ii, 366.

[8] Heffter, A., *Ergebn. Physiol.*, 1905, iv, 242.

[9] Reale, E., Abstr., *Centr. klin. Med.*, 1891, xii, 487, quoted from *Jahresber. Tierchem.*, 1891, xxi, 401 (orig., *Gaz. clin.*, 1890, i, 2).

[10] Jonescu, D., *Biochem. Z.*, 1906, i, 399.

[11] Siegfried, M., and Zimmerman, R., *Biochem. Z.*, 1911, xxxiv, 471.

[12] Siegfried and Zimmerman, *Biochem. Z.*, 1912, xlvi, 210.

[13] Baumann, E., *Z. physiol. Chem.*, 1886, x, 123.

receiving only water for 2 days was given 2 gm. of calomel on the 2nd day. As on the 4th day ethereal sulfates were still present in the urine, 2 gm. of calomel were again given. The urine of the 2 following days was free of ethereal sulfates, and gave no test for phenol or indole. On the 6th day, the dog received 5 gm. of tyrosine, but no formation of ethereal sulfates resulted; neither was there an increase of hydroxy acids.

Wohlgemuth[14] fed 8 gm. of tyrosine to a rabbit and was able to recover a little less than 2 gm. of it. Dakin[15] reports no increase of phenolic substances after giving tyrosine or phenylalanine. After feeding tyrosine to cats, he recovered by crystallization from the urine very small amounts of tyrosine. He found also that with smaller doses no unchanged tyrosine could be recovered in the urine. Brieger[16] could find no tyrosine in the feces or urine after giving 20 gm. of tyrosine to a man weighing about 50 kilos. He did, however, find an increase in the phenols, and although the patient was constipated for 2 days after taking the tyrosine, Brieger held that the increase in phenols could not be due to constipation alone. Brieger's figures follow:

Day.	Phenol. gm.
1	0.0159
2	0.0225
3	0.0223
4	0.0182
20 gm. of tyrosine ingested in two portions.	
5	0.0493
6	0.1576
7	0.0851
8	0.0609
9	0.0348

Results obtained with animals and with man have led Folin[1] to conclude that the excretion of total phenol products in the urine appears to be much greater than is indicated by the phenol figures previously recorded in the literature. He shows further that the phenols are not quantitatively converted into conjugated phenols, so that the detoxication process involved in such conjugations appears to furnish only a partial protection against the toxic effect of the phenol products formed by putrefaction in the intestinal tract. Finally, the total phenol excretion tends to vary directly, but not proportionally, with the protein intake.

Some of the work described in this summary has been confirmed by our findings, while some has not. The value of our figures lies in the fact that

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[14] Wohlgemuth, J., *Ber. chem. Ges.*, 1905, xxxviii, 2064.

[15] Dakin, H. D., *J. Biol. Chem.*, 1910–11, viii, 28.

[16] Brieger, L., *Z. physiol. Chem.*, 1878–79, ii, 241; 1879, iii, 134.

they have been obtained by a more accurate method, that of Folin,[17] which permitted not only a definite quantitative determination, but also the study of the relationship between the free and the conjugated phenols.

Methods.

For phenols, free and conjugated, the technique of Folin[17] was used, bearing in mind the following precautions: (a) In determining total phenols, a few glass beads should be used to prevent the liquid in the test-tube from over-boiling, during the heating just prior to placing the tube in the boiling water bath. (b) The phosphotungstic phosphomolybdic acid reagent is prepared by boiling the various chemicals with 750 cc. of water instead of 75 cc., the amount stated in Folin's description, which was an error. (c) The stock phenol solution from which the standard is prepared holds its strength for a long period of time, whereas the standard phenol solution itself deteriorates, and should therefore be prepared freshly every 4 or 5 days. (d) The color obtained with the standard solution is not absolutely blue, but has a greenish tinge. This defect is remedied by filtration, without in any way affecting the colorimeter reading. Therefore, after standing the required 20 minutes, the standard is filtered through inexpensive coarse filter paper,[18] and the colorimeter readings are made at once.

With these precautions we have found the method accurate, rapid, and easy of application.

EXPERIMENTAL.

In this investigation an effort has been made to determine the behavior of the animal body, with respect to the formation and elimination of phenols, under both normal and pathological conditions. Dogs were chosen as being best suited for the work. After a period of normal observation, the animals were operated upon,[19] and the desired pathological conditions produced. All operations were done under ether anesthesia. In all, eight operations were performed, two each of: (*1*) *Eck fistula*; (*2*) *intestinal obstruction*; (*3*) *exclusion of bile from the intestinal tract*; (*4*) *exclu-*

[17] Folin and Denis, *J. Biol. Chem.*, 1915, xxii, 305.

[18] Arthur H. Thomas Co., No. 27756, 125 mm.

[19] For the operative work, I am indebted to Dr. Max Minor Peet, of the Department of Surgical Research.

sion of pancreatic juice from the intestinal tract. The technique employed in each operation was as follows:

1. Eck Fistula.—An artificial anastomosis between the portal vein and the inferior vena cava, with ligation of the portal vein at the hilus of the liver, was made according to the technique described by Peet.[20]

2. Intestinal Obstruction.—Almost complete obstruction of the ileum was obtained about 6 inches from the ileocecal valve by detaching from the external sheath of the rectus a strip about ½ inch wide and 1 inch long, passing it through the mesenteric attachment around the gut, overlapping, and suturing the two ends of the fascia firmly together.

3. Exclusion of Bile from the Intestinal Tract.—The common bile duct was tied in three places, cut between the ligatures, and part of the omentum sewed in between the cut ends to prevent a possible reunion.

4. Exclusion of Pancreatic Juice from the Intestinal Tract.—In one animal two ducts were found, while in another, three ducts were present. Each duct was divided between ligatures and part of the omentum interposed between the cut ends, for the reason just mentioned.

Plan of Investigation.

The general plan of the investigation was to study the daily output of urinary and fecal phenols in dogs under the conditions outlined. The elimination of phenols, under the influence of fasting, catharsis, and phlorhizin, was also studied. Inasmuch as only minimal amounts of phenol were found in the feces—amounts that could have no bearing on the final results—these determinations were dispensed with.

The dogs, kept in metabolism cages, were fed on a standard diet, calorifically sufficient, and containing about 1 gm. of nitrogen per kilo so as to make certain of an excess of food in the intestine. This diet consisted of meat, lard, bread crumbs, sugar, salt, and sufficient bone ash to insure a well formed stool. The food was mixed with 400 cc. of water, and enough additional water was given separately to bring the total daily intake up to about 600 cc. The animals were placed on this diet 3 or 4 days before the

[20] Peet, M. M., *Ann. Surg.*, 1914, lx, 601.

beginning of an experiment. Female dogs only were used, the urine being collected by catheter every morning at the same hour and diluted to 1,000 cc. For a time the volume of urine excreted was noted, but this was later omitted for the reason that no particular relationship was seen between the volume of urine and the amount of phenols eliminated, except as noted in Table I. The body weight was recorded daily immediately after catheterization. Tyrosine was administered *per os*, either suspended in water or mixed with the food. Phenol (Merck reagent) and *p*-cresol (Kahlbaum) were given *per os* in water.

TABLE I.

Eck Fistula. Dog 15–58.

Influence of Water and Eck Fistula on Phenol Excretion.

Date.	Volume of urine.	Total N.	Phenols.				Weight.	Remarks.
			Free.	Total.	Free.	Conjugated.		
1915	cc.	gm.	gm.	gm.	per cent	per cent	kg.	
Oct. 19	600	8.9	0.158	0.181	87	13	12.4	
" 20	910	9.7	0.165	0.192	86	14	12.4	
" 21	695	6.1	0.163	0.188	87	13	12.5	
" 22	910	9.7	0.165	0.190	87	13	12.7	
" 23	745	10.4	0.163	0.188	87	13	12.6	
" 24	550	9.9	0.161	0.186	87	13	12.5	
" 25	415	9.9	**0.173**	**0.203**	**85**	**15**	12.7	Water intake reduced to 300 cc.
" 26	405	9.8	0.177	0.205	86	14	12.7	
" 27	345	9.9	0.173	0.203	85	15	12.8	
" 28	450	9.9	0.174	0.203	86	14	12.9	
" 29	450	9.9	0.172	0.201	86	14	12.9	
" 30	440	9.8	0.172	0.203	85	15	13.0	
" 31	455	9.9	0.170	0.200	85	15	13.0	
Nov. 10								Eck fistula.
" 11	400	10.2	**0.187**	**0.203**	**92**	**8**	12.7	Post-operative condition good.
" 12	250	9.9	**0.186**	**0.204**	**92**	**8**	12.6	
" 13	350	10.3	**0.188**	**0.204**	**92**	**8**	12.9	
" 14	360	10.6	**0.197**	**0.209**	**94**	**6**	13.1	
" 15	590	10.7	**0.205**	**0.213**	**96**	**4**	13.2	
" 16	570	10.6	**0.199**	**0.211**	**94**	**6**	13.1	
" 17	480	10.6	**0.200**	**0.213**	**94**	**6**	13.0	

Eck Fistula.

Experiment A-1.—Dog 15–58 (Table I). This experiment showed several interesting points. The output of phenols from day to day was quite constant, as was also the case in all of our subsequent work. Withdrawing water caused a drop in the volume of urine with a consequent rise in the output of phenols. However, it was seen that where the urine varied normally from day to day, there was little or no effect upon the phenols. After Eck fistula, the free phenols represented from 92 to 96 per cent of the total, the amount of the latter being practically unchanged. On Nov. 17, because of the presence of blood in the urine, the dog was placed on a kennel diet.

Experiment A-2.—Dog 15–58 (Table II). 2 weeks later, on Dec. 1, the regular diet was resumed, the experiment being started Dec. 6. For some reason, which we have been unable to explain —unless it is that the food is digested more rapidly—the absolute amount of total phenols was decreased in the second period, but the free phenols still represented from 96 to 98 per cent of the total. 1 gm. of phenol was fed *per os* with the result that 68.7 per cent of the amount ingested was eliminated. Both free and conjugated phenols were increased. The free phenols represented only 40 per cent of the total, indicating that the large dose of phenol had called forth, to an increased extent, the protective mechanism of the body. A second feeding gave similar results. Feeding 1 gm. of *p*-cresol resulted in the elimination of 50.6 per cent of the amount given. Here also both free and conjugated phenols were increased. The free phenols represented only 31 per cent of the total. Repetition of this feeding gave somewhat similar results. In this experiment and in all subsequent ones, it was noted that the phenol and *p*-cresol administered were eliminated entirely within 24 hours.

The results obtained on feeding tyrosine have a peculiar interest. For example, Dog 15–58 (Table II), receiving 400 gm. of meat—equivalent to 3.20 gm. of tyrosine,[21] or 1.65 gm. of phenol —eliminated daily a total of only 0.159 gm. of phenol. However, on feeding 5 gm. of tyrosine—equivalent to 2.57 gm. of phenol—there was a rise in both free and total phenols, while the

[21] Folin and Denis, *J. Biol. Chem.*, 1912, xii, 246.

TABLE II.

Eck Fistula. Dog 15–58.

Influence of Phenol, p-Cresol, and Tyrosine on Phenol Excretion after Eck Fistula.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1915	gm.	gm.	gm.	per cent	per cent	kg.	
Dec. 6	9.0	0.143	0.147	97	3	13.9	Cage water resumed.
" 7	9.0	0.147	0.152	97	3	13.9	
" 8	9.1	**0.334**	**0.836** (**0.787**)* (**78.7%**)	**40**	**60**	14.0	1.000 gm. phenol given in water *per os*.
" 9	9.1	0.152	0.156	97	3		
" 10	9.1	0.153	0.157	97	3	14.2	
" 11	9.2	**0.222**	**0.517** (**0.360**) (**72.0%**)	**41**	**59**	14.2	0.500 gm. phenol given in water *per os*.
" 12	9.3	0.154	0.159	97	3	14.2	
" 13	9.5	0.156	0.159	98	2	14.4	
" 14	9.5	**0.188**	**0.600** (**0.441**) (**50.6%**)	**32**	**68**	14.5	1.000 gm. *p*-cresol (0.870 gm. phenol) given in water *per os*.
" 15	9.4	0.154	0.159	97	3	14.5	
" 16	9.5	**0.174**	**0.541** (**0.381**) (**43.8%**)	**34**	**66**	14.5	*p*-Cresol fed as on Dec. 14.
" 17	9.7	0.154	0.157	98	2	14.5	
" 18	10.3	**0.420**	**0.603** (**0.446**) (**17.7%**)	**70**	**30**	14.7	5.000 gm. tyrosine (2.570 gm. phenol) given in water *per os*.
" 19	9.6	0.152	0.156	97	3	14.7	

* The figures in the first parenthesis represent "extra" phenols eliminated; those in the second parenthesis denote the output of "extra" phenols in terms of percentage of the amount ingested.

"extra" phenol eliminated was only 0.446 gm., or 17.7 per cent, of the amount ingested. *In other words—and it was to be expected—tyrosine, as present in meat, did not give rise to as much phenol as did free tyrosine. Similarly, phenol, administered as tyrosine or p-cresol, did not give rise to as much phenol as did native*

phenol. It was observed that tyrosine, like phenol and *p*-cresol, is eliminated entirely within 24 hours. A greater conjugation was also seen, though not as large as that produced by phenol and *p*-cresol. A slight rise in the nitrogen elimination was also noted. At no time was it possible to demonstrate unchanged tyrosine, either in the urine or the feces.

Experiment A-3.—Dog 15–58 (Table III). 1 gm. of phlorhizin rubbed up in 10 cc. of olive oil was injected subcutaneously

TABLE III.

Eck Fistula. Dog 15–58.

Influence of Phlorhizin, Fasting, and Catharsis on Phenol Excretion after Eck Fistula.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1915	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Dec. 19	9.6	0.152	0.156	97	3	14.7	
" 20	13.1	**0.203**	**0.277**	**73**	**27**	14.8	1 gm. phlorhizin.
" 21	15.5	**0.217**	**0.281**	**77**	**23**	14.7	1 " "
" 22	13.9	**0.171**	**0.210**	**82**	**18**	14.4	Sugar in urine Dec. 20–23, inclusive.
" 23	9.9	0.154	0.159	97	3	14.3	Animal placed on kennel diet till Dec. 31, when regular diet was resumed.
1916							
Jan. 5	10.2	0.150	0.158	95	5	14.7	
" 6	5.2	0.077	0.111	70	30	14.9	Animal fasting; 500 cc. of water given in cage.
" 7	4.8	0.068	0.091	74	26	14.4	
" 8	4.4	0.068	0.092	74	26	13.9	
" 9	4.0	0.068	0.091	75	25	13.6	
" 10	3.6	0.067	0.090	74	26	13.4	
" 11	3.5	0.066	0.091	73	27	13.2	
" 12							3 gm. calomel given; cage urine contaminated; bladder urine contained phenols.
" 13	3.4	**0.066**	**0.089**	**74**	**26**	12.9	
" 14	3.3	**0.066**	**0.091**	**73**	**27**	12.7	3 gm. calomel given.
" 25	3.0	**0.065**	**0.089**	**74**	**26**	11.5	Animal still fasting.

on 2 successive days. The result was an increase in both total and free phenols, the latter representing 73 per cent of the total. Sugar appeared in the urine, just as it did later, under similar cir-

TABLE IV.

Eck Fistula. Dog 16–56.

Influence of Phenol, p-Cresol, and Tyrosine on Phenol Excretion before and after Eck Fistula.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1916	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
May 9	10.3	0.176	0.235	75	25	12.3	
" 10	9.6	**0.385**	**0.782** (**0.547**) (**54.7%**)	**49**	**51**	12.4	1.000 gm. phenol given in water *per os.*
" 11	10.1	0.179	0.241	74	26	12.4	
" 12	10.3	**0.435**	**0.606** (**0.365**) (**14.2%**)	**72**	**28**	12.5	5.000 gm. tyrosine (2.57 gm. phenol) mixed with food.
" 13	10.2	0.176	0.233	75	25	12.6	
" 14	10.2	**0.210**	**0.556** (**0.323**) (**37.1%**)	**38**	**62**	12.5	1.000 gm. *p*-cresol (0.870 gm. phenol) given in water *per os.*
" 15							Eck fistula; post-operative condition good.
" 16	10.1	**0.208**	**0.250**	**83**	**17**	11.9	
" 17	10.2	**0.177**	**0.208**	**85**	**15**	12.1	
" 18	10.3	**0.384**	**0.555** (**0.347**) (**13.5%**)	**69**	**31**	12.3	Tyrosine fed as on May 12.
" 19	10.1	**0.350**	**0.815** (**0.607**) (**60.7%**)	**43**	**57**	12.5	Phenol fed as on May 10.

cumstances, in a normal dog. This is in accord with the observations of Sweet and Ringer,[22] who found that upon giving phlorhizin to a dog with Eck fistula, a glycosuria resulted quite comparable to that occurring in a normal dog.

[22] Sweet, J. E., and Ringer, A. I., *J. Biol. Chem.*, 1913, xiv, 135.

In fasting, as was to be expected, there was a diminution in both total and free phenols, the latter amounting to 74 per cent of the total. A greater ability to conjugate was shown, but no initial fall and subsequent rise in the phenol excretion as described by Herter[4] and Müller[5] was noted. Neither was it possible, by giving calomel, to rid the urine entirely of phenols. In this connection, it is interesting to recall that Baumann,[13] owing perhaps to the inadequacy of the prevailing methods, could get no test for phenols after administering calomel to a fasting dog.

Experiment G.—Dog 16–56 (Table IV). The results of this experiment are in accord with those obtained in the previous Eck fistula dog. After the operation there was a tendency for the total phenol elimination to decrease, due perhaps to a more rapid rate of digestion. The influence of tyrosine before and after the operation is about the same—if anything, for the reason just mentioned, less total phenols are obtained from the same amount of tyrosine after the operation than before.

Influence of Tyrosine, Phenol, and p-Cresol on Phenol Excretion in Normal Dogs.

Experiments K and M.—Dogs 15–74 and 16–20 (Table V). In general the findings here corroborated those of Experiment G.

TABLE V.

Normal Dog 15–74.

Influence of Tyrosine on Phenol Excretion.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1915	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Nov. 21	12.1	0.187	0.217	·86	14	14.3	
" 22	7.4	0.187	0.217	86	14	14.4	
" 23	12.1	**0.447**	**0.610** (**0.393**) (**15.3%**)	**73**	**27**	14.4	5.000 gm. tyrosine (2.570 gm. phenol) given in water *per os.*
" 24	12.8	0.187	0.217	86	14	14.7	
" 25	13.2	0.189	0.217	87	13	14.8	

TABLE V—*Concluded.*

Dog 16–20.

Influence of Phenol and p-Cresol on Phenol Excretion.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1916	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Feb. 20	10.5	0.158	0.192	83	17	11.1	
" 21	6.3	0.164	0.195	84	16	11.3	
" 22	10.7	0.167	0.196	85	15	11.1	
" 23	10.5	0.169	0.196	86	14	11.1	
" 24	10.2	0.166	0.192	86	14	11.1	
" 25	9.9	**0.181**	**0.647** (**0.455**) (**52.3%**)	**41**	**59**	11.2	1.000 gm. *p*-cresol (0.870 gm. phenol) given in water *per os*.
" 26	10.1	0.157	0.192	82	18	11.3	
" 27	10.2	**0.417**	**0.886** (**0.694**) (**69.4%**)	**47**	**53**	11.4	1.000 gm. phenol given in water *per os*.

Exclusion of Pancreatic Juice from the Intestinal Tract.

Experiment B-1.—Dog 15–63 (Table VI). The normal figures recorded here are in accord with those of Tables II and V.

Experiment B-2.—Dog 15–63 (Table VII). This experiment corroborates fully Experiment A-3 (Table III). While the results in the latter experiment were observed in a dog with Eck fistula, those of the present experiment were noted in a normal dog.

Experiment B-3.—Dog 15–63 (Table VIII). Several differences were noted between the results of this experiment and those of Experiment B-2 (Table VI). After the operation, the total amount of phenols eliminated was increased, resulting in a greater conjugation. On administering tyrosine, a greater percentage was eliminated as phenols. Undoubtedly this was due to the fact that the absence of pancreatic juice from the intestinal tract retarded the processes of digestion. The administration of phenol and of *p*-cresol resulted in a lessened elimination; *i.e.*, with phenol, there was a drop from 75.8 per cent to 51.1 per cent of

TABLE VI.

Exclusion of Pancreatic Juice. Dog 15–63.

Influence of Phenol, p-Cresol, and Tyrosine on Phenol Excretion before Operation.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1915	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Dec. 6	12.7	0.189	0.223	85	15	15.1	
" 7	12.6	0.192	0.222	86	14	15.1	
" 8	13.1	**0.442**	**0.980** (**0.758**) (**75.8%**)	**45**	**55**	15.2	1.000 gm. phenol given in water *per os.*
" 9	12.9	0.195	0.228	86	14		
" 10	13.1	0.195	0.226	86	14	15.3	
" 11	12.9	**0.411**	**0.944** (**0.718**) (**71.8%**)	**44**	**56**	15.4	Phenol fed as on Dec. 8.
" 12	13.0	0.195	0.228	86	14	15.2	
" 13	13.9	0.195	0.224	87	13	15.5	
" 14	14.2	**0.215**	**0.651** (**0.427**) (**49.2%**)	**33**	**67**	15.5	1.000 gm. *p*-cresol (0.870 gm. phenol) given in water *per os.*
" 15	14.1	0.195	0.225	87	13	15.5	
" 16	14.2	**0.218**	**0.606** (**0.381**) (**43.8%**)	**36**	**64**	15.4	Cresol fed as on Dec. 14.
" 17	13.5	0.195	0.223	87	13	15.6	
" 18	14.5	**0.455**	**0.625** (**0.402**) (**15.6%**)	**73**	**27**	15.6	5.000 gm. tyrosine (2.57 gm. phenol) given in water *per os.*
" 19	13.3	0.192	0.222	87	13	15.7	

TABLE VII.

Exclusion of Pancreatic Juice. Dog 15–63.

Influence of Phlorhizin, Fasting, and Catharsis on Phenol Excretion before Operation.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1915	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Dec. 19	13.3	0.192	0.222	87	13	15.7	
" 20	14.8	**0.200**	**0.268**	**75**	**25**	15.8	1 gm. phlorhizin.
" 21	15.5	**0.212**	**0.278**	**76**	**24**	15.7	1 " "
" 22	14.5	0.192	0.232	83	17	15.6	Sugar in urine Dec. 20–23, inclusive. Animal placed on kennel diet till Dec. 31, when regular diet was resumed.
1916							
Jan. 5	13.4	0.188	0.224	84	16	16.0	
" 6	4.6	0.090	0.130	70	30	16.0	Animal fasting; 500 cc. of water given in cage.
" 7	3.6	0.081	0.107	76	24	15.7	
" 8	3.5	0.080	0.106	76	24	15.4	
" 9	3.2	0.080	0.106	76	24	15.2	
" 10	3.1	0.079	0.106	75	25	14.9	
" 11	3.3	0.081	0.107	76	24	14.8	
" 12							3 gm. calomel given; cage urine contaminated; bladder urine contained phenols.
" 13	3.1	**0.080**	**0.106**	**76**	**24**	14.3	
" 14	3.0	**0.081**	**0.106**	**76**	**24**	14.1	3 gm. calomel given.
" 26	11.1	**0.184**	**0.223**	**83**	**17**	15.2	Animal on regular diet since Jan. 15.

TABLE VIII.

Exclusion of Pancreatic Juice. Dog 15–63.
Influence of Phenol, p-Cresol, and Tyrosine on Phenol Excretion after Operation.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total	Free.	Conjugated.		
1916	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Jan. 26	11.1	0.184	0.223	83	17	15.2	
" 28							Pancreatic ducts cut; dog in good condition.
Feb. 6	9.4	**0.200**	**0.289**	**69**	**31**	15.5	
" 7	10.0	**0.203**	**0.293**	**69**	**31**	15.4	
" 8	9.7	0.202	0.286	71	29	15.3	
" 9	9.9	**0.660**	**0.845** (**0.559**) (**21.7%**)	**78**	**22**	15.4	5.000 gm. tyrosine (2.57 gm. phenol) mixed with food.
" 10	9.5	0.202	0.289	70	30	15.3	
" 11	9.5	0.200	0.293	68	32	15.5	
" 12	10.1	**0.279**	**0.632** (**0.339**) (**38.9%**)	**44**	**56**	15.5	1.000 gm. *p*-cresol (0.870 gm. phenol) given in water *per os*.
" 13	8.7	0.203	0.289	70	30	15.3	
" 14	8.5	**0.423**	**0.800** (**0.511**) (**51.1%**)	**53**	**47**	15.3	1.000 gm. phenol given in water *per os*.
" 15	8.6	0.200	0.286	70	30	15.2	

the amount fed; with *p*-cresol, there was a drop from 49.2 per cent to 38.9 per cent of the amount given. With both substances there is, however, an increased conjugation because of the increased formation of phenols.

Experiment H.—Dog 16–55 (Table IX). The results of this experiment need no further comment since they corroborate the previous findings as recorded in Tables VI and VIII.

TABLE IX.

Exclusion of Pancreatic Juice. Dog 16–55.
Influence of Phenol, p-Cresol, and Tyrosine on Phenol Excretion before and after Section of Pancreatic Ducts.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1916	gm.	gm.	gm.	per cent	per cent	kg.	
May 9	9.5	0.151	0.200	75	25	11.6	
" 10	9.8	**0.417**	**0.847** (**0.647**) (**64.7%**)	**49**	**51**	11.6	1.000 gm. phenol given in water *per os*.
" 11	9.6	0.156	0.204	76	24	11.7	
" 12	9.6	**0.208**	**0.594** (**0.390**) (**44.8%**)	**35**	**65**	11.8	1.000 gm. cresol (0.870 gm. phenol) given in water *per os*.
" 13	9.7	0.153	0.200	76	24	11.8	
" 14	9.8	**0.440**	**0.625** (**0.425**) (**16.5%**)	**70**	**30**	11.8	5.000 gm. tyrosine (2.570 gm. phenol) mixed with food.
" 15							Pancreatic ducts cut; dog in poor condition.
" 16	9.6	**0.175**	**0.250**	**70**	**30**	11.3	
" 17	9.4	**0.173**	**0.255**	**68**	**32**	11.0	
" 19							Animal chloroformed.

Intestinal Obstruction.

Experiment C.—Dog 16–6 (Table X). The results obtained before the operation are in accord with those found in normal dogs. After the operation, it is worthy of note that although the animal ate practically nothing, the formation and elimination of phenols rose to a high level, the free phenols representing only 56 per cent of the total. In other words, the conjugation, due to the larger amounts of phenols present, was increased just as though a dose of phenol had been ingested. It was noted also that on giving phenol after the operation, only 51 per cent was eliminated, while before the operation the output was 64 per cent. This is explained on the ground that digestion is markedly retarded. In this respect the results were somewhat similar to

TABLE X.

Intestinal Obstruction. Dog 16–6.

Influence of Phenol, p-Cresol, and Tyrosine on Phenol Excretion before and after Intestinal Obstruction.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1916	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Feb. 23	9.6	0.175	0.227	77	23	10.9	
" 24	9.5	0.171	0.222	77	23	11.1	
" 25	9.7	**0.199**	**0.548** (**0.326**) (**37.4%**)	**36**	**64**	11.1	1.000 gm. *p*-cresol (0.870 gm. phenol) given in water *per os.*
" 26	9.6	0.173	0.221	78	22	11.2	
" 27	9.5	**0.400**	**0.862** (**0.641**) (**64.1%**)	**46**	**54**	11.1	1.000 gm. phenol given in water *per os.*
" 28	9.7	0.174	0.221	79	21	11.3	
" 29	9.8	**0.429**	**0.605** (**0.384**) (**15.0%**)	**71**	**29**	11.4	5.000 gm. tyrosine (2.57 gm. phenol) mixed with food.
Mar. 1	9.9	0.173	0.219	79	21	11.3	
" 9							Intestine obstructed.
" 10							Urine contaminated.
" 11							" "
" 23	9.8	**0.263**	**0.477**	**55**	**45**		Dog defecated hard stool for first time since operation.
" 24	7.2	**0.245**	**0.442**	**56**	**44**	9.6	Ate only part of diet.
" 25	6.4	**0.238**	**0.403**	**58**	**42**	9.2	No food given Dec. 25 and Dec. 26.
" 26	4.9	**0.500**	**0.914** (**0.511**) (**51.1%**)	**55**	**45**	8.9	1.000 gm. phenol given in water *per os.*

those obtained before and after the exclusion of pancreatic juice from the intestinal tract. On March 27, when the animal was chloroformed, the intestine was found to be dilated to about 100 times the normal capacity for about 3 feet above the obstruction; this dilatation continued in a lesser degree up to the jejunum. The jejunum and duodenum were normal. It would appear that almost complete obstruction had been obtained.

Experiment F.—Dog 15–63 (Table XI). The findings in this experiment were not as marked as those in Experiment C, due perhaps to the fact that the amount of obstruction secured in this case was not as great as in Dog 16–6. However, there was a definite rise in the formation and elimination of phenols, though nothing unusual was to be noted as a result of the combination of pancreatic insufficiency and intestinal obstruction. Apparently the increased phenol formation was not sufficient to cause an increase in the conjugation, the free phenols representing almost the same per cent of the total both before and after the operation.

TABLE XI.

Dog 15–63.

Influence of Intestinal Obstruction, Combined with Pancreatic Insufficiency, on Phenol Excretion.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1916	gm.	gm.	gm.	per cent	per cent	kg.	
Apr. 8	12.7	0.190	0.270	70	30	13.9	
" 9	12.9	0.200	0.263	76	24	14.2	
" 10	13.2	**0.498**	**0.860** (**0.597**) (**59.7%**)	**58**	**42**	14.2	1.000 gm. phenol given in water *per os.*
" 11	12.9	**0.357**	**0.588** (**0.325**) (**37.3%**)	**61**	**39**	14.3	1.000 gm. *p*-cresol (0.870 gm. phenol) given in water *per os.*
" 12	12.8	0.187	0.257	72	28	14.3	
" 13	12.7	0.195	0.263	74	26	14.3	
" 17							Intestine obstructed.
" 19	7.5	**0.227**	**0.316**	**71**	**29**	13.7	Dog did not eat.
" 20	6.5	**0.543**	**0.870** (**0.554**) (**55.4%**)	**63**	**37**	13.5	" " " " 1.000 gm. phenol given in water *per os.* No lard in diet beginning Apr. 22.
May 3	10.9	**0.223**	**0.307**	**72**	**28**	13.7	
" 4	11.4	**0.555**	**0.855** (**0.548**) (**54.8%**)	**65**	**35**	13.8	Phenol fed as on Apr. 20.
" 5	11.3	**0.227**	**0.316**	**72**	**28**	13.7	

Exclusion of Bile from the Intestinal Tract.

Experiment D.—Dog 16–39 (Table XII). The figures obtained before the operation are similar to those in a normal dog. After the operation, however, a striking difference was noted. *Although*

TABLE XII.

Exclusion of Bile. Dog 16–39.
Influence of Phenol, p-Cresol, and Tyrosine on Phenol Excretion before and after Operation.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1916	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Mar. 27	13.6	0.161	0.215	75	25	19.6	
" 28	13.8	**0.365**	**0.758** (**0.543**) (**54.3%**)	**48**	**52**	19.9	1.000 gm. phenol given in water *per os.*
" 29	13.7	0.167	0.217	76	24	20.3	
" 30	13.9	**0.447**	**0.613** (**0.396**) (**14.8%**)	**72**	**28**	20.5	5.000 gm. tyrosine (2.570 gm. phenol) mixed with food.
" 31	13.9	**0.189**	**0.590** (**0.373**) (**42.8%**)	**32**	**68**	20.7	1.000 gm. *p*-cresol (0.870 gm. phenol) given in water *per os.*
Apr. 1							Bile duct cut; dog in good condition.
" 2	10.9	**0.167**	**0.208**	**80**	**20**	20.5	Bile in urine; little food eaten.
" 3	10.7	**0.330**	**0.642** (**0.434**) (**43.4%**)	**52**	**48**	20.6	Phenol fed as on Mar. 28; dog ate very little; bile in urine.
" 7							Dog gave birth.
May 4	5.8	**0.238**	**0.291**	**82**	**18**	11.8	Animal markedly jaundiced.
" 5	6.4	**0.291**	**0.513** (**0.222**) (**25.5%**)	**57**	**43**	11.5	Cresol fed as on Mar. 31.
" 6	6.5	**0.263**	**0.329**	**80**	**20**	11.4	
" 9	6.7	**0.629**	**0.839** (**0.510**) (**19.8%**)	**75**	**25**		Tyrosine fed as on Mar. 30.

there was an increase in the formation and elimination of phenols, the free phenols represented 80 per cent of the total, as against 75 per cent before the operation. In all other experiments, an increase in the phenol formation was accompanied by a decrease in the output of free phenols, figured as per cent of total, while in this experiment and in the following one the reverse was the case. Of the ingested phenol and *p*-cresol, 43.3 per cent of the former and 25.5 per cent of the latter were eliminated after the operation as against 54.3 and 42.8 per cent, respectively, before. Feeding tyrosine caused an increased conjugation, the free phenols dropping from 75 to 72 per cent before the operation, and from 80 to 75 per cent after. It was observed, however, that only 14.8 per cent of the ingested

TABLE XIII.

Exclusion of Bile. Dog 16–41.
Influence of Phenol, p-Cresol, and Tyrosine on Phenol Excretion before and after Operation.

Date.	Total N.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
1916	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*kg.*	
Apr. 8	11.3	0.151	0.191	79	21	9.6	
" 9	11.1	0.152	0.186	82	18	9.7	
" 10	11.2	**0.477**	**0.782** (**0.596**) (**59.6%**)	**61**	**39**	9.7	1.000 gm. phenol given in water *per os.*
" 11	11.2	**0.347**	**0.568** (**0.382**) (**43.8%**)	**61**	**39**	9.7	1.000 gm. cresol (0.870 gm. phenol) given in water *per os.*
" 12	11.1	0.147	0.182	81	19	9.8	
" 13	9.0	**0.440**	**0.596** (**0.314**) (**12.2%**)	**74**	26	9.9	5.000 gm. tyrosine (2.57 gm. phenol) given in water *per os.*
" 14							Bile duct cut; animal in poor condition.
" 15	5.6	**0.098**	**0.123**	**80**	**20**	8.9	No food eaten; bile in urine.
" 16	5.2	**0.096**	**0.120**	**80**	**20**	8.9	No food eaten; bile in urine.
" 17							Animal chloroformed.

tyrosine was eliminated before the operation as against 19.8 per cent after. This was similar to the result obtained with tyrosine after excluding the pancreatic juice, and, as in the latter case, may be explained on the basis of delayed digestion.

Experiment E.—Dog 16–41 (Table XIII). The results of this experiment were not as clean cut as they might be, for the animal refused to eat and was rather sick. On autopsy the bile duct, up to the point tied, was greatly enlarged.

DISCUSSION.

The formation of phenolsulfuric acids is one example of the ability of the body to convert poisonous substances into harmless compounds. It is, however, an open question as to whether this power of the body is sufficient for all purposes. Since the time of Baumann it has been believed that phenols were quantitatively converted into harmless phenol esters; hence phenols were estimated on the basis of conjugated sulfates, whereas our figures corroborate the statement made by Folin[1] that the greater part of the phenols are excreted unconjugated.

It has been thought that the free phenols are harmful to the body, and this must be due primarily to an increase of total phenols, with its accompanying increase of free phenols. For example, it is seen in normal cases that the absolute amount of free phenols represents from 75 to 85 per cent of the total. On the other hand, where there is a rise in total phenols, whether due to ingested phenols or to some pathological condition, the free phenols, although increased absolutely, represent from 30 to 70 per cent of the total. In other words, the absolute amount of free phenols should be taken into consideration together with the percentage in considering their deleterious influence.

With one exception, an increase in the formation of phenols, whether normal or pathological, resulted in an increased conjugation, though the reverse might have been expected. It would appear that the protective mechanism of the body responded to the greater stimulus. The exception noted was in the case where bile was excluded from the intestinal tract. Here there was an increased phenol formation accompanied by a decreased conjugation. It would seem, therefore, that the bile plays some part in assisting the liver in its conjugating function.

There is a greater conjugation after feeding *p*-cresol than after giving phenol. In view of the fact that *p*-cresol makes up the larger part of the urinary phenols, it seems plausible to assume that when it is introduced into the body it is more easily conjugated. Hence on feeding this substance the elimination of free phenols rises only very little.

The high percentage of free phenols prevailing after Eck fistula was to be expected, inasmuch as the liver, which is the main seat of conjugation, has been cut out of the circulation. In spite of this, upon the ingestion of phenol there is an increased conjugation, showing that other organs, when necessary, can take up the work of the liver in this respect.

Lewin[6] held that the increased protein breakdown accounted for the increased phenol formation when phlorhizin was given. While it is true that phlorhizin causes a protein breakdown, it is highly improbable that this is the reason for an increased phenol production. Regarding the fate of phlorhizin in the body, it has been shown[23] that when injected, part of it is eliminated as a combined glucoronic acid while another part apparently undergoes further change; also, unchanged phlorhizin can be found for some time in the blood and tissues. In view of all this, when it is considered that phlorhizin contains two benzene radicals, and that benzene when fed[1] gives rise to phenol, it seems likely that it is the phlorhizin *per se* which is converted into phenol.

The results obtained show, among other things, that gastrointestinal disturbances, in which it is reasonable to assume that there is an increase in the formation of phenol, and also certain liver diseases, where it is supposed that the mechanism for the detoxication of phenols has been impaired, offer a field for investigation, for, as Folin[1] points out, and as this work proves, the phenols can be taken as an index of intestinal putrefaction.

SUMMARY.

The results of this investigation, in addition to confirming the findings of Folin,[1] bring to light some interesting observations.

A. 1. The elimination of phenols from day to day is quite constant.

[23] Von Fürth, O., Chemistry of Metabolism, Philadelphia, 1916, 280.

2. Withdrawing water from the diet causes an increased phenol elimination.

3. After Eck fistula, the free phenols represent as high as 97 per cent of the total, the latter, as also the former, exhibiting a tendency to decrease.

4. After intestinal obstruction, there is an increase in both free and total phenols, the former constituting as low as 55 per cent of the latter.

5. In pancreatic insufficiency, there is an increase in both free and total phenols with an accompanying decrease in the percentage of free phenols.

6. After excluding the bile, both free and total phenols increase, but with an increase in the percentage of free phenols.

B. 1. The feeding of phenol and *p*-cresol results normally, with but slight variations, in the elimination of about 65 per cent and 40 per cent respectively.

2. After Eck fistula, about the same excretion is noted.

3. After intestinal obstruction, pancreatic insufficiency, and exclusion of bile, there is in both cases a drop in the amount excreted.

C. 1. The feeding of tyrosine results normally in an excretion of about 14 per cent, as phenols.

2. After Eck fistula, practically the same amount is eliminated.

3. After exclusion of bile and pancreatic juice, about 20 per cent of ingested tyrosine is eliminated.

D. Feeding of any of the three substances causes an increase in the conjugation at all times—*p*-cresol to a greater degree than phenol, and the latter to a greater extent than tyrosine.

E. Tyrosine, phenol, and *p*-cresol, fed in amounts of 5 gm., 1 gm., and 1 gm. respectively, were all eliminated within 24 hours.

F. No unchanged tyrosine could be demonstrated in the urine or feces.

G. Fasting reduces the phenols to a low level; the injection of phlorhizin during fasting causes an increase in the output of phenols.

H. It is impossible to free the urine entirely of phenols by the use of calomel.

I. The bile appears to have some influence on the conjugating function of the liver.

J. The phenols can be taken as an index of intestinal putrefaction.

THE INFLUENCE OF INGESTED CARBOHYDRATE, PROTEIN, AND FAT ON THE BLOOD SUGAR IN PHLORHIZIN DIABETES.

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(Received for publication, June 14, 1916.)

INTRODUCTION.

By introducing glucose solution into a normal animal, an increase in blood sugar occurs which lasts but a short time and the normal level for blood sugar is then reestablished. The destination of the absorbed sugar is not yet known. The possibilities are that, after passing through the intermediary stages of metabolism it is burned; or it is distributed in the body as glucose; or is converted into glycogen and deposited in the tissues; or is eventually converted into fat. In the phlorhizinized dog the above mentioned possibilities are limited to the temporary deposition of glucose or glycogen since the ingested glucose reappears practically quantitatively in the urine. On studying the velocity of the elimination of the "extra glucose," it was found that of 16 gm. of glucose ingested, 94 per cent was excreted in the urine during the first 5 hours, and that the maximum amount appeared during the 2nd and 3rd hours.[1] The endogenous glucose derived from glycine and alanine was eliminated almost as rapidly as the isoglucogenic quantity of exogenous glucose.

The purpose of this paper is to show the influence of ingested carbohydrate, protein, and fat on the blood sugar in phlorhizinized animals; the blood sugar being determined in hourly periods to compare the rate of absorption of glucose, both of exogenous and of endogenous origin, to the rate of elimination determined by means of "extra glucose" mentioned above.

[1] Csonka, F. A., *J. Biol. Chem.*, 1915, xx, 539.

Method.

Dogs were used in these experiments. After 2 days of fasting, the dogs were given daily injections for 4 days of 1.2 gm. of phlorhizin suspended in 10 cc. of olive oil. The glucose solution was introduced through a stomach tube; the other foods were eaten by the dog, the time required being less than 2 minutes. The 50 gm. of gelatin were dissolved in 200 cc. of warm water, mixed with 2 gm. of Liebig's extract, cooled, and given in the solid form. The blood was taken from the ear veins. The blood sugar was determined immediately after the blood was cooled to room temperature, according to the Lewis-Benedict method.[2] The ligation of the kidneys was performed through an abdominal incision, under ether anesthesia;[3] both the renal vessels and the ureter were included in the ligature. At the conclusion of the experiments, the dogs were killed; the liver was removed, weighed, ground, and 100 gm. were taken for glycogen, determined after the method of Pflüger.[4] The muscles of the hind legs were removed and prepared for the determination of glycogen, using 100 gm. of the ground muscle.

EXPERIMENTAL PART.

Fisher and Wishart[5] found that 50 gm. of glucose given *per os* to dogs were absorbed in 4 hours; the blood showed only a slight increase in blood sugar, and after 2 hours the excess had entirely disappeared. The increase of glycogen content of the liver was not found until absorption was complete; *i.e.*, in 4 hours.

My intention was to repeat the experiment, thinking that by using the phlorhizinized dog, in which there is a very low glycogen content in the tissue, the glucose absorbed during the 4 hours might be detected in the muscle in the form of glycogen. But after a preliminary experiment and a subsequent one, in which the kidneys were ligated for the purpose of giving greater oppor-

[2] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[3] I desire to thank Drs. C. C. Guthrie and C. C. Hartman for their kindness in performing this operation on the dogs.

[4] Pflüger, E., *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1910, ii, 1070.

[5] Fisher, G., and Wishart, M. B., *J. Biol. Chem.*, 1912–13, xiii, 49.

tunity for deposition of the absorbed glucose, the anticipated results were not found. The proposed experiment was therefore abandoned.

Table I shows the glycogen content of the liver and muscle after 50 gm. of glucose in 150 cc. of water were given.

TABLE I.

Experiment.	Date.	Dog.		Weight of liver.	Glycogen of			Remarks.
					Liver.		Muscle.	
		No.	Weight.		Total.			
			kg.	*gm.*	*gm.*	*per cent*	*per cent*	
1	Apr. 27, 1915	22	13.7	390	0.039	0.010	0.242	Dog phlorhizinized 9 days; killed 2½ hrs. after feeding.
2	Dec. 2, 1915	24	10.2	268	0.760	0.289	0.330	Dog phlorhizinized 6 days. Kidneys ligated. Killed 4 hrs. after feeding.
3	Jan. 11, 1916	26	11.8	313	0.046	0.015	0.595	Normal dog; kidneys ligated; killed 6 hrs. after feeding.

The amount of glycogen present in muscle and liver cannot be considered increased, as this amount falls within the limits reported for the phlorhizinized dogs.[6]

Table II shows the changes of the blood sugar content following the ligation of the renal vessels and ureters and the subsequent

TABLE II.

	Blood sugar.	
	A. Experiment 2.	B. Experiment 3.
	per cent	*per cent*
Before operation	0.058	0.075
2 hrs. after kidney ligation	0.151	0.099
4 " " ligation; 2 hrs. after glucose feeding	0.290	0.076
6 " " " 4 " " " "	0.246	0.084
8 " " " 6 " " " "		0.090

[6] Lusk, G., *Ergebn. Physiol.*, 1912, xii, 315.

ingestion of 50 gm. of glucose, (A) in the phlorhizinized dog, and (B) in the normal dog.

A marked hyperglycemia is found in the phlorhizinized dog, but in the normal dog the blood sugar remains practically at the normal level. In both cases the stomach contents showed a positive glucose reaction. In the former (A) it was impossible to determine the glucose quantitatively on account of the loss of the stomach contents by killing. In the latter case (B), in 310 cc. of stomach contents, the glucose amounted to 15.92 gm.

In Table III are collected the results of experiments performed on phlorhizinized dogs fed glucose, meat, gelatin, and lard.

TABLE III.

Experiment.	Date.	Dog.		Fed.	Blood sugar (in percentages).						
					Before feeding.	After feeding.					
		No.	Weight.			1st hr.	2nd hr.	3rd hr.	4th hr.	6th hr.	8th hr.
			kg.								
4	Mar. 7	27	12.5	20 gm. glucose in 200 cc. water.	0.060	0.181	0.192	0.113	0.070	0.088	
5	May 1	27*	12.9	50 gm. glucose in 150 cc. water.	0.066	0.173	0.191	0.175	0.204	0.106	0.083
6	June 2	28	9.5	50 gm. gelatin.	0.061	0.085	0.092	0.101	0.081	0.085	0.078
7	Mar. 8	27	12.2	200 gm. beef meat.	0.058	0.066	0.088	0.077	0.075	0.071	
8	May 3	27*	11.3	500 gm. beef meat.	0.069	0.102	0.119	0.098	0.097	0.095	0.083
9	" 31	28	10.0	100 gm. lard.	0.074	0.075	0.069	0.059	0.056	0.058	
10	" 16	30	20.6	143 " "	0.064	0.074	0.069	0.067	0.062	0.053	0.058

* Second phlorhizination.

Except in the case of dogs fed lard (which represents fat in general) all show an increase in blood sugar; *but the increase following the ingestion of 20 gm. of glucose, for example, is greater than that following the ingestion of 200 gm. of meat, though they represent approximately isoglucogenic quantities.*[7]

[7] Compare Janney, N. W., and Csonka, F. A., *J. Biol. Chem.*, 1915, xxii, 203.

DISCUSSION AND CONCLUSION.

Following the ingestion of 20 gm. of glucose the blood sugar reached the maximum at the 2nd hour, and declined to the original level at the 4th hour. In an earlier experiment,[1] it was shown that of 16 gm. of glucose given to a phlorhizinized dog, 94 per cent was excreted during the first 5 hours as "extra glucose." The curve of the blood sugar content runs parallel with the curve of the "extra glucose." Since the elimination of ingested glucose is practically complete and the curve of blood sugar runs parallel

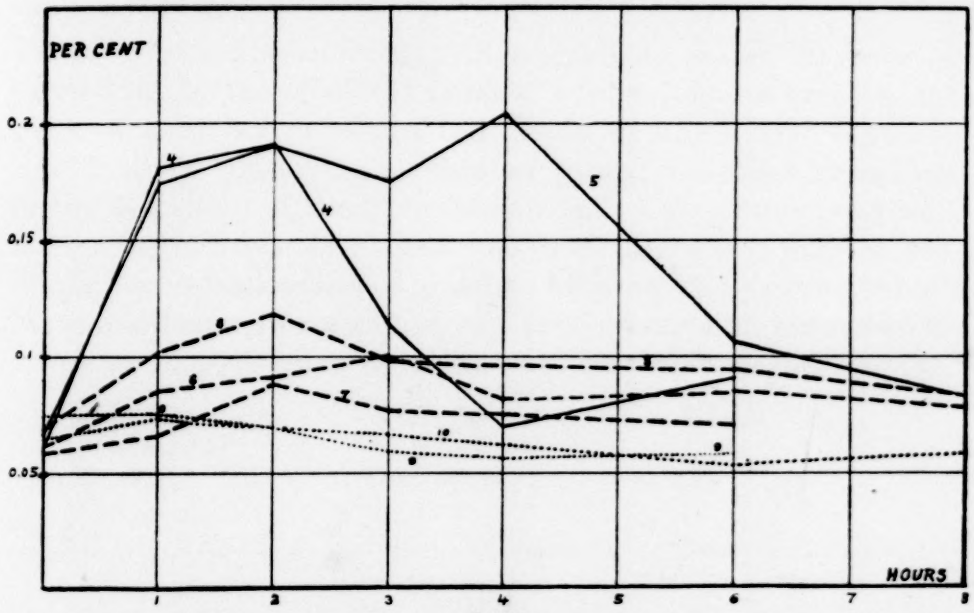

FIG. 1. The curves represent the blood sugar content after the ingestion of carbohydrate (———), protein (– – – – –), and fat (.....). The number of the curve corresponds with the number of the experiment.

to that of "extra glucose," the conclusion is reached that no glucose was deposited between the periods of absorption and elimination. The only difference observed in the blood sugar content after the ingestion of large quantities of glucose in a more concentrated solution (50 gm. of glucose in 150 cc. of water) is that the hyperglycemia is maintained for a longer period of time (Fig. 1). That the blood sugar is increased after ingestion of protein is apparent from the curve which shows that the endogenous glucose derived from meat protein and gelatin appears in

the blood as glucose—at least, partially so—since the isoglucogenic quantities of ingested endogenous and exogenous glucose should give the same increase of blood sugar. The ingestion of fat, which does not produce "extra glucose," did not cause any appreciable increase in blood sugar.

RESEARCHES ON PYRIMIDINES.

LXXIX. THE SYNTHESIS OF THYMINE-4-ALDEHYDE.

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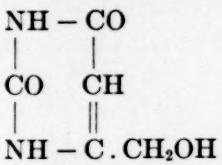
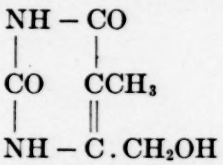
(Received for publication, June 6, 1916.)

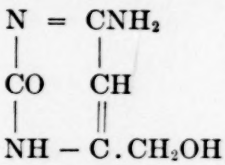
The pyrimidine-nucleoside investigations which have been carried on in the Sheffield Laboratory by Johnson and his coworkers have been confined to a study of uracil and thymine combinations containing monatomic primary alcohol groupings in the 4-position of the pyrimidine ring. Two simple representatives have thus far been described in the literature; namely, the simple mono-nucleosides of uracil and thymine as represented by Formulas I and II respectively. The corresponding nucleoside of the aminopyrimidine cytosine (III) has not been prepared.



I



II

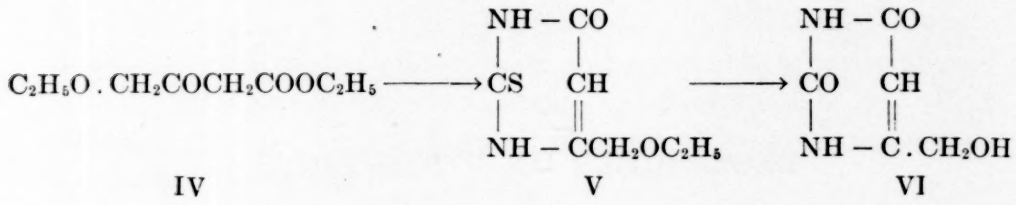


III

The only method of synthesis which has proven feasible thus far for the preparation of pyrimidines leading to nucleoside combinations of the above type is that involving the condensation of thiourea with certain β-ketone esters. Ethyl γ-ethoxyacetoacetate (IV) is the simplest representative of the class of ketone esters utilized in this work, and by interaction with thiourea leads to the formation of the 2-thiopyrimidine (V). The latter is easily converted into the uracil-nucleoside (VI), as has been shown in a previous paper by Johnson and Chernoff.[1]

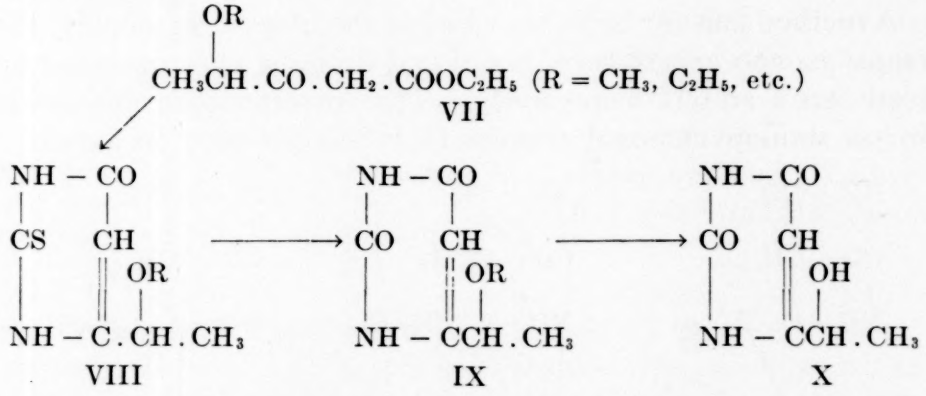
* Part of a Dissertation presented to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy, 1916.

[1] Johnson, T. B., and Chernoff, L. H., *J. Am. Chem. Soc.*, 1914, xxxvi, 1742; 1913, xxxv, 585; *J. Biol. Chem.*, 1913, xiv, 307.



IV V VI

In order to extend the application of our method and obtain higher homologues of these simple nucleosides, or pyrimidine combinations containing secondary alcohol groups, we have prepared several representatives of a new type of β-ketone esters corresponding to VII. Such combinations condense normally with thiourea giving 2-thiopyrimidines (VIII), and the latter are desulfurized easily by digestion with chloroacetic acid with production of their corresponding ethers represented by IX. To our surprise, however,

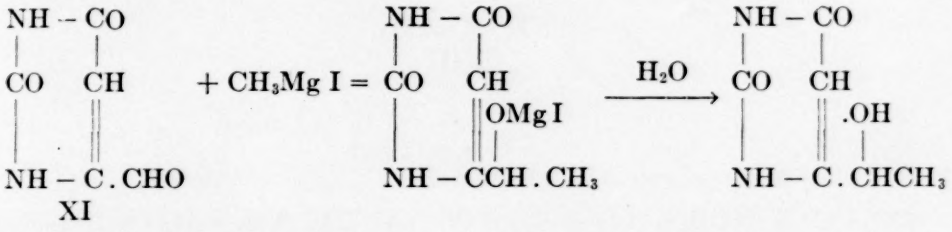


VII VIII IX X

when we attempted to hydrolyze these ethers (IX) to their corresponding alcohols (X), we obtained very abnormal and unexpected results. Products were obtained which possessed none of the properties of the desired pyrimidine nucleosides, and the data thus far obtained lead us to the conclusion that pyrimidine combinations of this type (X) cannot be made by this method. The experimental evidence supports the assumption that such secondary alcohol combinations are transformed by hydrolysis into representatives of an entirely different class of cyclic compounds.[2]

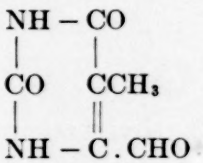
[2] The results of this investigation will soon be published in a paper from this laboratory by Treat B. Johnson and Sidney E. Hadley.

In the light of these new and unexpected results it was therefore necessary to direct our attention to the preparation of pyrimidines carrying an aldehyde group in the 4-position of the ring; in other words, to synthesize the aldehydes of uracil and thymine (XI and XII). Such combinations should theoretically interact with Grignard's reagents with formation of the desired pyrimidine nucleosides containing secondary alcohol groups. A transformation of this character would be represented by the following equation:[3]



XI

A method has now been developed whereby such aldehyde combinations can be synthesized easily and in quantity. The complete synthesis of uracil aldehyde (XI) has already been described by the writers[4] and in this paper we shall give a description of the synthesis and properties of the corresponding aldehyde of thymine (XII) and also several of its derivatives.



XII

The nearest approach to an aldehyde synthesis recorded in the pyrimidine literature, previous to the developments in this laboratory, is that involving a reaction described by Behrend.[5] He made the interesting observation that amidomethyluracil (XIII) is transformed very smoothly into the oxime of diazouracil-4-aldehyde (XIV) by the action of nitrous

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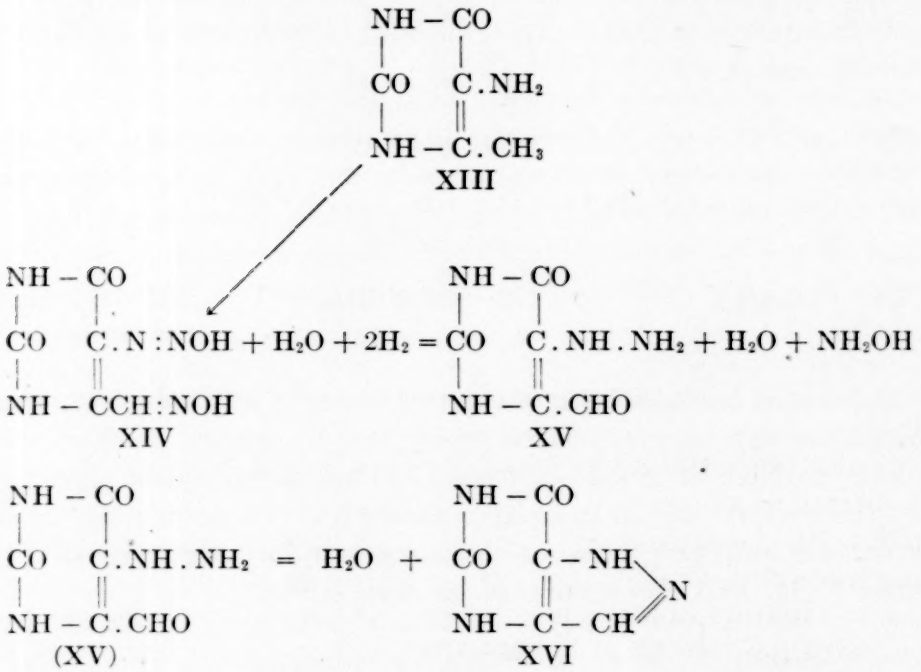
[3] The investigation of the behavior of Grignard's reagents towards pyrimidine aldehydes will be taken up this coming year.

[4] Johnson, T. B., and Cretcher, L. H., *J. Am. Chem. Soc.*, 1915, xxxvii, 2144.

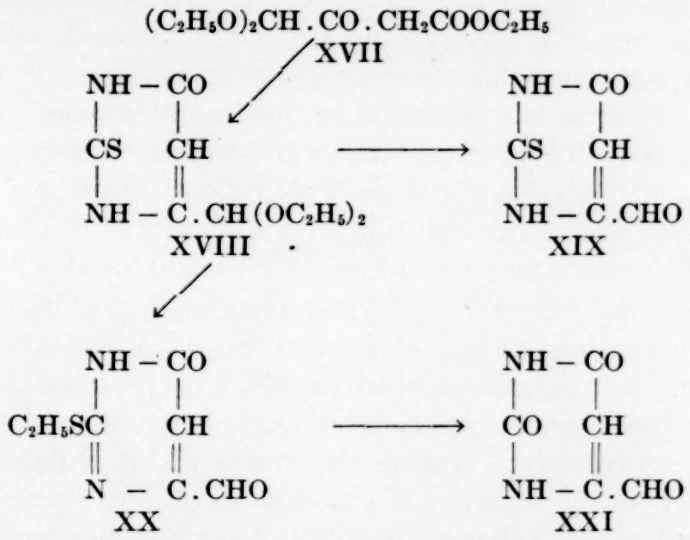
[5] Behrend, R., *Ann. Chem.*, 1888, ccxlv, 213.

acid. This oxime was isolated in a crystalline condition and was characterized by its remarkable stability. On reduction with stannous chloride in hydrochloric acid solution it was transformed into a compound isomeric with xanthine (XVI). Behrend called this substance *Isoxanthine* and suggested the following expression as a possible explanation of the mechanism of the transformation. The intermediate product—hydrazinouracil-aldehyde (XV)—was not isolated.



The first pyrimidine aldehydes actually isolated were 2-thiouracil-4-aldehyde (XIX), 2-ethylmercapto-6-oxypyrimidine-4-aldehyde (XX), and uracil-4-aldehyde (XXI), which were synthesized by Johnson and Cretcher.[4] Their method of introducing the aldehyde group into the pyrimidine ring differs from any which has hitherto been employed for the synthesis of heterocyclic aldehydes. It is based on the observation that esters of the type of ethyl γ-diethoxyacetoacetate (XVII) condense normally with thiourea giving the corresponding diethylacetals of 2-thiopyrimidine aldehydes. The ester (XVII) interacts, for example, with thiourea forming the acetal (XVIII) which can be transformed easily by hydrolysis into the aldehyde of 2-thiouracil (XIX). The aldehyde combinations described proved to be extremely reactive.

They interacted normally with phenylhydrazine, and possessed a remarkable capacity of adding water, which could not be removed without heating at high temperatures.



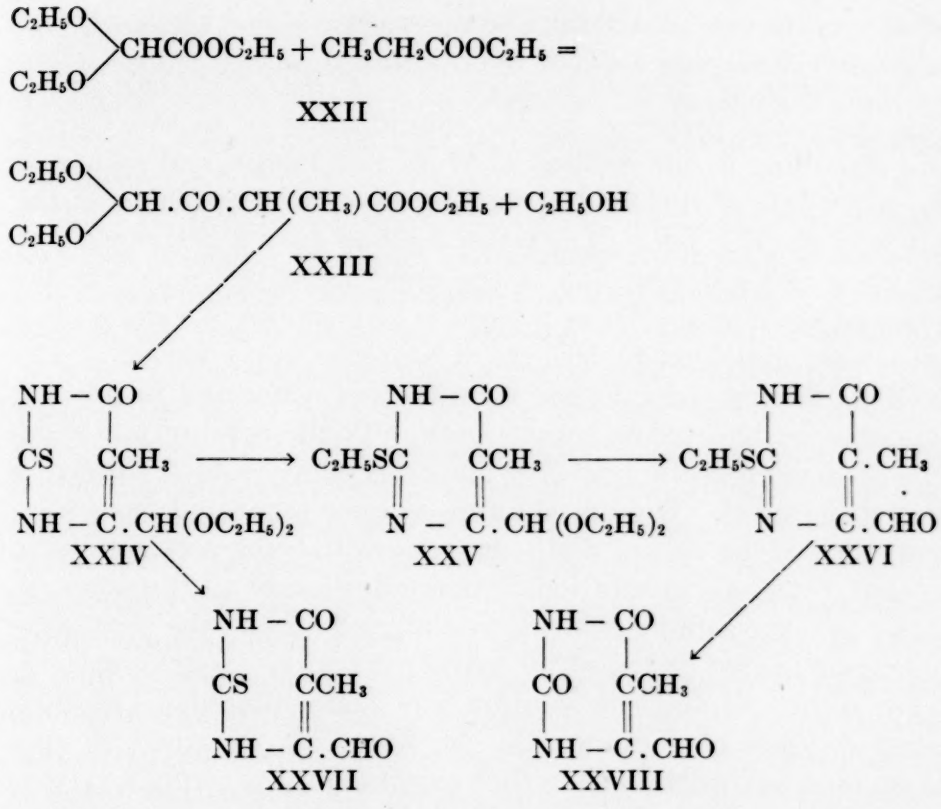
Dakin and Dudley[6] have shown that the ketone ester—ethyl γ-diethoxyacetoacetate (XVII)—interacts normally with methyl iodide, in the presence of sodium ethylate, giving the corresponding methyl derivative (XXIII). We find that the same ketone ester is easily obtained in good yield by application of a Claisen condensation with ethyl diethoxyacetate and ethyl propionate (XXII). When this substance is brought into alcohol solution with thiourea and sodium ethylate it behaves in a similar manner as its lower homologue (XVII) and interacts with the thiourea forming the acetal of 2-thiothymine-4-aldehyde (XXIV). The yield is excellent. This acetal behaves exactly as its uracil analogue except that it is less soluble in hydrochloric acid solution. It dissolves in alkaline solutions without decomposition and is reprecipitated unaltered from such solutions by the addition of acids. When warmed with acids, both mineral and organic, it is easily hydrolyzed with formation of 2-thiothymine-4-aldehyde (XXVII). This compound crystallizes from dilute hydrochloric acid with a molecule of water and is characterized by its remarkable stability.

[6] Dakin, H. D., and Dudley, H. W., *J. Chem. Soc.*, 1914, cv, 2453.

The 2-thioacetal (XXIV) undergoes alkylation quantitatively with ethyl bromide in alcohol solution, and in the presence of the required amount of sodium ethylate, giving the mercaptopyrimidine (XXV). This acetal is extremely unstable in the presence of acids and is even hydrolyzed by acetic acid, in the cold, with formation of the corresponding mercaptopyrimidine aldehyde (XXVI). This new aldehyde is a beautiful crystalline substance, but is not so active a reducing agent as the corresponding oxygen and sulfur aldehydes. It does not reduce Fehling's solution and reduces silver nitrate in alkaline solution with difficulty.

When the mercaptopyrimidine (XXV) is heated with hydrochloric acid, not only the acetal grouping is affected but the mercapto group undergoes hydrolysis as well, with liberation of ethylmercaptan, and thymine-4-aldehyde (XXVIII) is formed. It is necessary, however, to digest for a long time to remove the mercapto radical completely. During the course of this prolonged treatment the aqueous solution is not discolored but remains bright yellow, and no amorphous or resinous decomposition products are formed. This behavior is quite different from that observed during the hydrolysis of the corresponding mercapto-6-oxypyrimidine-4-aldehyde.[4] Uracil aldehyde is much more sensitive to the action of acids than thymine aldehyde (XXVIII). Thymine aldehyde reduces Fehling's solution, but does not undergo oxidation when exposed to the air, nor did it oxidize in hydrochloric acid solution when the latter was exposed to the atmosphere for 2 weeks.

The tendency to combine with the solvent from which it is crystallized was observed in the case of thymine aldehyde as well as with uracil aldehyde. The thymine derivative crystallizes from aqueous solution with one molecule of water and when it separates from alcohol carries one molecule of this reagent. Both forms are easily dissociated on heating. The changes described above are represented by the following formulas:



The normal class reactions, which are generally characteristic of combinations containing the aldehyde group, can be applied successfully with the three pyrimidines XXVI, XXVII, and XXVIII. All combine with phenylhydrazine giving phenylhydrazones and likewise interact with hydroxylamine forming the corresponding oximes. The latter compounds are formed in good yield and should be of value for further synthetical work. Thiothymine-4-aldehyde (XXVII) and thymine-4-aldehyde (XXVIII) interact smoothly with aniline with formation of their corresponding anils. The aldehyde group in the pyrimidine (XXVI) undoubtedly interacts with aniline in a similar manner, but it was impossible to control the reaction, so that the corresponding mercaptopyrimidine-anil could be isolated. The mercapto group of the pyrimidine is removed at the same time with production of ethylmercaptan. The investigation of pyrimidine aldehydes will be continued.

EXPERIMENTAL PART.

Preparation of Ethyldiethoxyacetate. $(C_2H_5O)_2CH.COOC_2H_5$.—This ester was prepared by the esterification of diethoxyacetic acid according to the method of Wohl and Lange[7] and also from the silver salt of this acid by boiling an ether suspension of the salt with ethyl iodide. The latter method gave a better yield of the ester and a description of one experiment will illustrate the procedure followed. Three molecular proportions of sodium (56.2 gm.) were dissolved in 800 cc. of absolute ethyl alcohol. The flask containing the ethylate solution was connected to a reflux condenser and heated on a water bath. To this solution one molecule of dichloroacetic acid (105 gm.) was slowly added through a dropping funnel. Sodium chloride at once separated from solution; the sodium salt of diethoxyacetic acid, being very soluble in alcohol, remained in solution. After addition of all the dichloroacetic acid the sodium chloride was filtered off and the clear solution again heated on the water bath for 3 hours. Sodium chloride again separated and the solution was again filtered. The combined residues of sodium chloride, when dry, weighed 95 gm., an amount corresponding to that required for the completed reaction. The alcohol was removed under reduced pressure, a dark colored pasty mass remaining in the flask. This crude sodium salt was dissolved in 150 cc. of water and the solution filtered. The solution was transferred to a large beaker, cooled with ice water, and a cold solution containing 145 gm. of silver nitrate (in 200 cc. of water) gradually added. There was at once precipitated a voluminous, non-crystalline mass of the silver salt of diethoxyacetic acid which was filtered free from water by suction. The filtrate was evaporated to dryness under reduced pressure and the residue combined with the main portion of the salt. This was then dried in a vacuum desiccator over concentrated sulfuric acid.

Conversion of the Silver Salt into Ethyldiethoxyacetate.—The dry salt obtained above was suspended in 400 cc. of dry ether and 145 gm. of ethyl iodide were added gradually through a dropping funnel. After all the iodide had been added the containing flask was heated in a water bath for 4 hours. The solution was filtered

[7] Wohl, A., and Lange, M., *Ber. chem. Ges.*, 1909, xli, 3612.

and the silver iodide washed several times with dry ether. After removal of the ether, the almost colorless oil was distilled *in vacuo*. The yield was 93 gm. of pure ester, boiling between 94 and 98° at 19 mm. pressure. This amount corresponds to 65 per cent of the theoretical.

Ethyl α-Methyl-γ,γ-diethoxyacetoacetate (XXIII).—This ester was first prepared by Dakin and Dudley[6] by heating the sodium salt of ethyl diethoxyacetoacetate with methyl iodide. The ester used in this investigation was prepared by the action of sodium on a mixture of ethyl diethoxyacetate and ethyl propionate. The proportions taken were as follows: 65 gm. of diethoxyacetic ester, 110 gm. of ethyl propionate, and 24 gm. of sodium wire. All of the diethoxy ester and one-half of the propionic ester were mixed in a flask, connected with a reflux condenser, and heated to about 80°. One-half of the sodium was then gradually added. After about 3 hours the sodium had all reacted and the remainder of the propionic ester was added, after which the remainder of the sodium was introduced into the flask in small amounts at a time, as before, the heating being continued during the entire process. After the last of the sodium was added the temperature of the mixture was kept at 80° for 4 hours longer or until the sodium had practically disappeared. The mixture was then allowed to cool and ice water was added to dissolve the sodium salt of the β-ketone ester. The solution was transferred to a large separatory funnel and the unaltered esters were removed by extraction with ether. Much ether was now added and the lower aqueous layer made acid to litmus with cold dilute hydrochloric acid, and the layers separated. After three extractions with ether the water layer became colorless. The ether solution was finally washed with dilute sodium carbonate solution and dried for 6 hours over desiccated sodium sulfate. After removal of the ether the ester was obtained as a red oil and was distilled in a vacuum. When the temperature of the bath was raised to 145–155°, at a pressure of 17 mm., a few gm. of liquid distilled over from 90–112°. This fraction consisted of unchanged esters not removed by ether. When oil ceased to come over the temperature of the bath was raised to 175°. 20 gm. of colorless oil now distilled at 130–140°, the pressure remaining constant at 17 mm. With the bath at 180° and the pressure at 18 mm. 40 gm. more of the oil

distilled over at the constant temperature of 140°. Dakin and Dudley's product boiled between 110–112° at a pressure of 4 to 6 mm. The total yield of purified ester was 60 gm., corresponding to 70 per cent of the theory, based on the quantity of the diethoxyacetic ester used. The yield obtained by Dakin and Dudley was also 70 per cent. This ester is colorless and has a pleasant odor.

0.1506 gm. substance gave 0.3130 gm. CO_2 and 0.1160 gm. H_2O.

	Calculated for $C_{11}H_{20}O_5$:	Found:
C	56.60	56.9
H	8.50	8.6

2-Thio-4-diethoxymethyl-5-methyl-6-oxypyrimidine (XXIV).— This compound is easily obtained in the following manner. 9 gm. of sodium were dissolved in 200 cc. of absolute alcohol and 40 gm. of thiourea dissolved in the resulting solution. Next 51 gm. of the β-ketone ester, described above, were added and the mixture was heated on a water bath for 6 hours, after which time it was transferred to a casserole and the alcohol evaporated by heating on a steam bath. The residue was light yellow. It was dissolved in about 300 cc. of water, cooled, and the solution acidified with acetic acid. On adding the acid a colorless oil was obtained which readily became crystalline when stirred. The product was colorless, weighed 50 gm., and partially melted at about 119°. There was some thiourea present and the melting point was not sharp. The entire amount was crystallized from hot 50 per cent alcohol from which it separated on cooling, in large blocks which melted between 119–120°. 33 gm. of pure pyrimidine were thus obtained and 6 gm. more on concentration of the mother liquor.

This pyrimidine is slightly soluble in water and very soluble in alcohol.

Calculated for $C_{10}H_{16}O_3N_2S$: N, 11.4. Found: N, 11.4.

In order to obtain this product in pure form it is not necessary that the β-ketone ester be isolated and purified by distillation. In fact, the reaction with thiourea was first brought about with the crude undistilled ketone ester. The method was as follows: The lower boiling esters present as impurities in the crude mixture

were removed by heating the containing flask to 150° in an oil bath at 17 mm. pressure. After liquid ceased to distil under these conditions the dark red oil remaining was condensed with thiourea under the exact conditions described above. In order to obtain the final product perfectly colorless it is necessary, however, to make several recrystallizations from alcohol.

2-Thio-5-methyl-6-oxypyrimidine-4-aldehyde (2-Thiothymine Aldehyde) (XXVII).—A quantitative yield of this aldehyde is obtained by hydrolysis of its acetal with hydrochloric acid. The acetal was dissolved in hot dilute acid (1:1) and the solution evaporated to dryness on a water bath. During the course of the evaporation the color of the solution gradually became bright yellow. The powder remaining after the evaporation was yellow and melted to a dark oil at 232° with effervescence. It is soluble in hot water, alcohol, and acetic acid, and is more soluble in dilute hydrochloric acid than in water. When crystallized from alcohol the aldehyde is colorless, but upon being heated again becomes yellow. After crystallization from alcohol the melting point is 232–233°. The aldehyde when prepared by evaporation to dryness with hydrochloric acid does not carry water of crystallization.

Calculated for $C_6H_6O_2N_2S$: N, 16.4. Found: N, 16.3.

When allowed to crystallize slowly from dilute hydrochloric acid the aldehyde contains one molecule of water of crystallization.

Calculated for $C_6H_6O_2N_2S.H_2O$: N, 14.8. Found: N, 14.6.

This aldehyde reduces an ammoniacal silver nitrate solution but not Fehling's solution.

Derivatives of 2-Thiothymine-aldehyde. Phenylhydrazone.—Thiothymine aldehyde was dissolved in hot dilute acetic acid and phenylhydrazine added to the solution. The hydrazone separated at once as a yellow crystalline mass. It crystallizes from glacial acetic acid in long prismatic needles which darken around 265° and melt at 287° with effervescence. The compound is insoluble in water and only sparingly soluble in alcohol.

Oxime.—0.6 gm. of sodium hydroxide was dissolved in 10 cc. of water, 1 gm. of aldehyde dissolved in the alkaline solution, and 0.4 gm. of hydroxylamine hydrochloride was added. The solution

was allowed to stand over night at room temperature. The sodium salt of the oxime separated as a white powder but was not filtered off. This salt was converted to the oxime by the addition of just sufficient glacial acetic acid to give an acid reaction to the solution when tested with litmus. After stirring and allowing to stand for a few minutes the oxime was filtered off and recrystallized from glacial acetic acid, from which it separated in hexagonal plates which decomposed at 233°. The yield was 1 gm.

Calculated for $C_6H_7N_3O_2S$: N, 22.7. Found: N, 22.4.

This oxime is difficultly soluble in hot alcohol and glacial acetic acid, and almost insoluble in hot water.

Anil.—To prepare this Schiff base 1 gm. of the thioaldehyde was mixed with 5 cc. of aniline in a test-tube, and the mixture heated in a water bath for several hours. The aldehyde did not dissolve in the hot aniline. The color of the reaction mixture soon became a bright yellow. Ether was then added, after cooling, to remove the excess of aniline, and the solid filtered off. The anil was obtained as a yellow compound, insoluble in water or alcohol, and difficultly soluble in hot glacial acetic acid, from which it separates in yellow, hexagonal plates which decompose at about 274°.

Calculated for $C_{12}H_{11}ON_3S$: N, 17.1. Found: N, 17.0.

2-Ethylmercapto-4-diethoxymethyl-5-methyl-6-oxypyrimidine (*XXV*).—1.9 gm. of sodium were first dissolved in 250 cc. of absolute alcohol, 20 gm. of the acetal of 2-thio-4-thymine aldehyde dissolved in the resulting solution, and 15 gm. of ethyl bromide finally added. The flask was connected to a reflux condenser and heated on the water bath until the reaction mixture became neutral to litmus. The sodium bromide was then filtered off and the alcohol solution concentrated to a volume of about 25 cc. Water was then added when a colorless oil was precipitated which assumed a crystalline form when stirred. This product was filtered off and dried over sulfuric acid. The yield of pure substance was 22 gm., or practically theoretical. This compound is very soluble in alcohol and glacial acetic acid, and sparingly soluble in water. It was crystallized from 50 per cent alcohol from which

it separated in long needles. The melting point of the pure product is 100°.

Calculated for $C_{12}H_{21}O_3N_2S$: N, 10.2. Found: N, 10.2.

2-Ethylmercapto-5-methyl-6-oxypyrimidine-4-aldehyde(XXVI).—This aldehyde was formed quantitatively from the corresponding acetal by dissolving the acetal in 50 per cent acetic acid and then evaporating the solution on a water bath. The compound is white in color, slightly soluble in hot water, and easily soluble in alcohol and acetic acid. It was purified by crystallization from alcohol and separated in long needles which melted at 186° to an oil.

This aldehyde does not reduce Fehling's solution and reduces an ammoniacal silver nitrate solution only on prolonged boiling.

Calculated for $C_8H_{10}N_2O_2S$: N, 14.1. Found: N, 14.0.

Derivatives of 2-Ethylmercapto-5-methyl-6-oxypyrimidine-4-aldehyde. Phenylhydrazone.—A small amount of the aldehyde was dissolved in dilute acetic acid and a few drops of phenylhydrazine were added to the hot solution. There was an immediate precipitation of a yellow crystalline hydrazone, which was purified by crystallization from alcohol. It softens at 201°, remaining in a semi-melted condition to 238° at which temperature it decomposes. The crystals are needles and show a tendency to aggregate in rosettes. This hydrazone is sparingly soluble in water but soluble in glacial acetic acid.

Oxime.—The procedure followed was identical to that described before for the formation of the oxime of 2-thiothymine aldehyde. The yield was 1 gm. The product was purified by crystallization from 60 per cent acetic acid and separated in the form of distorted, elongated, prisms which decomposed at about 235°. The compound is colorless, insoluble in alcohol and water, and very soluble in glacial acetic acid.

Calculated for $C_8H_{11}O_2N_3S$: N, 19.2. Found: N, 18.9.

Anil.—It was found impossible to prepare a Schiff base by heating this mercapto aldehyde with pure aniline or with aniline diluted with alcohol, the mercapto group being removed and ethylmercaptan liberated in both cases.

2,6-Dioxy-5-methylpyrimidine-4-aldehyde (*XXVIII*).—A practically quantitative yield of this pyrimidine was obtained by the hydrolysis of the acetal of 2-ethylmercapto-4-thymine aldehyde with hydrochloric acid. 15 gm. of the acetal were dissolved in about 300 cc. of hydrochloric acid (1: 1) and the solution heated on a water bath. Acid was added at intervals to replace that lost by evaporation and the heating continued until the odor of mercaptan was no longer noticed. The time required was about 48 hours. The solution was then evaporated to dryness, the residue being a white amorphous powder melting at 205°. It was purified by crystallization from dilute hydrochloric acid and separated, after standing some time, in the form of colorless distorted prismatic needles, arranged in rosettes. This compound was also observed to crystallize in hexagonal plates, from dilute hydrochloric acid.

Calculated for $C_6H_6O_3N_2.H_2O$: N, 16.3. Found: N, 16.1.

The water was removed by heating for 1 hour at 110°.

Calculated for $C_6H_6O_3N_2.H_2O$: H_2O, 10.5. Found: H_2O, 10.1.

When crystallized from absolute alcohol this aldehyde separated with one molecule of alcohol of crystallization. This alcohol was removed by heating for 1 hour at 110°.

Calculated for $C_6H_6O_3N_2C_2H_5OH$: C_2H_5OH, 23.0. Found: C_2H_5OH, 22.9.

This aldehyde reduced Fehling's solution and an ammoniacal silver nitrate solution. It is soluble in glacial acetic acid and water. The melting point is 212–213°.

Derivatives of Thymine Aldehyde. Phenylhydrazone.—A small amount of the aldehyde was dissolved in dilute acetic acid and the solution warmed. A few drops of phenylhydrazine were then added when this hydrazone separated at once in the form of yellow needles, often arranged in rosettes. The compound decomposes at about 272° without melting. It is slightly soluble in alcohol and water and soluble in glacial acetic acid.

Oxime.—In order to prepare this oxime the following proportions were used. 1 gm. of thymine aldehyde, 0.4 gm. of hydroxylamine hydrochloride, and 0.6 gm. of sodium hydroxide. The

procedure followed was the same as that described in the preparation of the oximes already spoken of and the yield was 1 gm. The product crystallizes from glacial acetic acid in elongated four-sided plates which decompose at about 257°. The compound is difficultly soluble in acetic acid and insoluble in alcohol and water.

Calculated for $C_6H_7O_3N_3$: N, 24.8. Found: N, 24.6.

Anil.—This Schiff base was prepared by dissolving a small amount of thymine aldehyde in aniline and boiling the resulting solution for a few minutes. On cooling, the anil separated from the aniline in the form of long yellow needles which were purified by recrystallization from glacial acetic acid. The compound softened at 265° and decomposed at 272°. This anil is insoluble in water and alcohol.

Calculated for $C_{12}H_{11}O_2N_3$: N, 18.2. Found: N, 18.2.

CEREBRONIC ACID.*

V. RELATION OF CEREBRONIC AND LIGNOCERIC ACIDS.

By P. A. LEVENE and C. J. WEST.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 16, 1916.)

In a recent publication from Thierfelder's laboratory Brigl[1] reported the results of some synthetic experiments which were initiated with the object of proving the Thierfelder-Brigl assumption of the structure of cerebronic acid. The article also contains critical remarks on our work on the same subject.

The starting point for the synthetic work of Brigl is the assumption that cerebronic acid has the structure of normal α-hydroxypentacosanic acid,

$$CH_3(CH_2)_{22}CHOHCOOH$$

In order to prove this contention he attempted to convert cerebronic acid into the corresponding hydrocarbon, and to prove the identity of this product with the synthetic *n*-pentacosan. Secondly, it was his intention to prepare synthetically the normal α-hydroxypentacosanic acid and to prove the identity of this synthetic product with *r*-cerebronic acid.

By a rather interesting process Brigl prepared *n*-pentacosan, but did not convert cerebronic acid into the paraffin. Hence he failed to accomplish the part of the task that rested on the study of the two paraffins.

Also by a very difficult and circuitous method he prepared normal α-hydroxypentacosanic acid. The comparison of the melting points of the synthetic acid with the *r*-cerebronic acid did not lead to a convincing conclusion.

* Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 381. Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913, xiv, 257; 1913, xv, 193; 1914, xviii, 477.

[1] Brigl, P., *Z. physiol. Chem.*, 1915, xcv, 161.

In a word, the synthetic products obtained by Brigl are interesting by themselves, but in the hands of Brigl they were of no service for the purpose of proving the structure of cerebronic acid.

The critical remarks on our work on cerebronic acid are directed not against the conclusions but against the method employed by us for the conversion of α-hydroxy fatty acids into the corresponding hydrocarbons. Thierfelder and Brigl discuss only the results of the publications of Levene and Jacobs of 1912 and of Levene and West of 1913. There the view was expressed that cerebronic acid had the structure of normal α-hydroxypentacosanic acid. The view was based on the fact that the hydrocarbon obtained from cerebronic acid melted at 53.5–54°C. This melting point was the one accepted by Marie[2] for *n*-pentacosan. Furthermore, cerebronic acid was converted into lignoceric acid which yielded a hydrocarbon melting at 51°C. This is the melting point accepted by Krafft[3] for *n*-tetracosan.

The hydrocarbon was obtained by the action of hydriodic acid in a sealed tube at 125°C. As already stated, Thierfelder and Brigl take exception to this method. Our own work, subsequent to the first two publications, convinced us that the method was better than the conclusions. The original view on the structure of cerebronic acid, as expressed by Levene and Jacobs and by Levene and West, was subsequently modified.

The present writers regard cerebronic acid to be the α-hydroxy derivative of the next higher homologue of lignoceric acid. Naturally we cannot accept the conclusions of Thierfelder and Brigl. However, we did not have absolute confidence in the hydriodic acid process for the conversion of fatty acids into paraffins. In fact, the original object of the hydriodic acid experiment was to convert cerebronic acid into pentacosanic acid. At the same time the work of Meyer, Brod, and Soyka[4] advanced the view that lignoceric acid had the structure of a branched chain tetracosanic acid. Since cerebronic acid is easily converted into lignoceric acid all the conclusions reached regarding the structure of one apply also to the other.

[2] Marie, T., *Ann. chim. phys.*, 1896, vii, 212; *Bull. Soc. chim.*, 1896, xv, 567.

[3] Krafft, F., *Ber. chem. Ges.*, 1882, xv, 1711.

[4] Meyer, H., Brod, L., and Soyka, W., *Monatschr. Chem.*, 1913, xxxiv, 1113.

We also realized the need of a revision of the melting points of the higher hydrocarbons and have adapted a process of preparation of the hydrocarbons which consisted of the following steps:

$$R\ COOC_2H_5 \rightarrow R\ CH_2OH \rightarrow R\ CH_2I \rightarrow R\ CH_3.$$

Every step of the process involved only very mild treatment. In this manner *n*-tetracosan and the isotetracosan corresponding to lignoceric acid were prepared.[5] The first had the melting point of 54°C. (given in literature 51.1°C.) and the second 51°C., identical with that previously obtained by the hydriodic acid process.

Furthermore, we prepared *n*-tetracosanic acid[6] and found for it a melting point of 87.5–88.0°C. (85.5–86.0°C. was given by Meyer, Brod, and Soyka). The melting point for lignoceric acid is 81°C.

Hence the structure of cerebronic acid seems to be fairly well correlated with that of lignoceric acid. For greater rigor of the proof we plan to convert lignoceric acid into an α-hydroxypentacosanic acid, which should be identical with cerebronic, and finally to compare the pentacosans obtained from cerebronic acid and the synthetic pentacosanic acid. We also contemplate converting lignoceric acid into isotricosanic acid, and by the malonic ester synthesis to arrive at pentacosanic acid which will serve both for preparation of the paraffin and of the α-hydroxy acid.

Another point of criticism is directed by Brigl against the assumption made by us for the explanation of the variability of the melting point of various samples and of various fractions of the α-hydroxypentacosanic acid obtained on hydrolysis of cerebrin. Our assumption was that the so called cerebronic acid is a mixture of optical isomers; Brigl holds the view that the isomerism of the two acids is structural. The objection of Brigl is based on the fact, first, that the difference in the melting of the optically active and inactive acids is in the neighborhood of 20°C., and second, on the fact that the synthetic normal hydroxypentacosanic acid melts 20° above the optically inactive natural acid.

The second proof of Brigl falls by itself since it has been proven that cerebronic acid does not belong to the series of normal acids.

[5] Levene and West, *J. Biol. Chem.*, 1914, xvii, 477.

[6] Levene, P. A., West, C. J., Allen, C. H., and van der Scheer, J., *J. Biol. Chem.*, 1915, xxiii, 71.

As to the first point of objection, it must be emphasized that we have not claimed to have established the correct melting point of either the active or the *dl*-forms of cerebronic acid. One always realizes the difficulty of any deductions from observations on mixtures of fatty acid. Our principal argument was that, regardless of the great difference in the melting points of different samples of the natural hydroxypentacosanic acids (generally mixtures), they all yield on oxidation a tetracosanic acid of the same sharp melting point of 81°C., an acid having all other properties of lignoceric acid. It is hard to believe that two isomeric tetracosanic acids have the same melting point when the α-hydroxy derivatives of their next higher homologues differ in their melting point by more than 20°C.

The view may be further substantiated by the fact that the same sample of cerebrin yields a cerebronic acid of different melting point and of different optical power depending upon the conditions of hydrolysis. A similar observation was made by Rosenheim.[7]

However, in our previous work no detailed record was kept of the rotatory power and of the melting points of the different samples of cerebronic acids which served for oxidation into lignoceric acid. In order to add validity to our earlier evidence we prepared several samples of cerebronic acid.

The first had a melting point of 99–100°, $[\alpha]_D^{20} = +2.6°$; the second, 86°, $[\alpha]_D^{20} = +1.5°$; and the third, 91–93°, $[\alpha]_D^{20} = +3.55.°$

The first two of these samples were oxidized with permanganate solution, and both yielded a tetracosanic acid melting sharply at 81°C.

Hence we feel that for the present there is not sufficient ground for a revision of the original view as to the difference in the melting points of the various cerebronic acids.

EXPERIMENTAL.

The cerebroside employed for the preparation of the cerebronic acid had a specific rotation of $[\alpha]_D^{20} = +10°$, and in solubility and other properties corresponded to Thierfelder's cerebron or Thudichum's phrenosin. The physical property of the fatty acid ob-

[7] Rosenheim, O., *Biochem. J.*, 1916, x, 148.

tained from the same material differed, depending on the conditions of hydrolysis.

I. Hydrolysis with Sulfuric Acid in an Autoclave.—100 gm. of cerebrin were heated with 1 liter of 3 per cent sulfuric acid (by weight) for 24 hours in an autoclave at 110–115°. The mixture of cerebronic acid and sphingosine was filtered off, the acid extracted with acetone, and purified through the sodium and lithium salts. This free acid was then recrystallized from acetone and melted at 80–85°. The rotation of the material varied from +1.2° to +1.5°.

By repeatedly fractionating the acid with lithium acetate in methyl alcoholic solution, using the insoluble fraction each time, a sample of acid was finally obtained which melted at 99–100° and had a specific rotation of 2.69°.

$$[\alpha]_D^{25} = \frac{3.3624 \times 0.16^\circ}{0.2002 \times 1.0} = 2.69^\circ$$

0.1008 gm. substance gave 0.2796 gm. CO_2 and 0.1160 gm. H_2O.

	Calculated for $C_{25}H_{50}O_3$:	Found:
C	75.33	75.32
H	12.65	12.88

This material was oxidized with alkaline potassium permanganate according to Levene and Jacobs and also with permanganate in acetone solution according to Levene and West. Recrystallized from acetone, analytically pure lignoceric acid, melting at 81°, was obtained.

0.1006 gm. substance gave 0.2886 gm. CO_2 and 0.1202 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	78.22
H	13.20	13.37

II. Hydrolysis with 20 Per Cent Hydrochloric Acid.—Cerebrin was heated with four parts of 20 per cent hydrochloric acid and one part of 95 per cent alcohol for 48 hours, as in the preparation of sphingine.[8] From the reaction product a cerebronic acid was isolated, which melted at 85–86°, and had a rotation of 1.5°.

[8] Levene, P. A., *J. Biol. Chem.*, 1916, xxiv, 74.

Upon oxidation with permanganate in acetone solution, lignoceric acid was obtained, melting at 80–81°.

0.1004 gm. substance gave 0.2882 gm. CO_2 and 0.1200 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	78.27
H	13.20	13.34

III. Hydrolysis with Methyl Alcohol-Sulfuric Acid.—Cerebrin, having a rotation of +10°, was hydrolyzed with methyl alcohol and sulfuric acid according to the directions given in earlier articles. The product, when purified through the sodium and lithium salts, and recrystallized from acetone, melted at 88–91°. After being twice extracted with low-boiling petroleum ether, the melting point was 91–93°. The activity of this sample was

$$[\alpha]_D^{20} = \frac{0.20° \times 5.857}{0.330 \times 1.0} = 3.55°$$

This acid was not oxidized. The experiment is reported to show the influence of the mode of hydrolysis on the physical properties of the resulting cerebronic acid.

THE DIALYSIS OF TRYPSIN AND THE PROTEOCLASTIC ACTION OF THE PROTEIN CLEAVAGE PRODUCTS.

By CASIMIR FUNK.

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(Received for publication, June 14, 1916.)

An extensive investigation as to the nature of trypsin was undertaken and published last year by Herzfeld.[1] In this work he stated that the cleavage products of various proteins possess a distinct hydrolytic action on proteins. On the basis of his experiments he attempted to demonstrate that the active properties of pepsin are mainly due to proteoses and those of trypsin to amino-acids. In his second paper[2] he subjected trypsin to dialysis and found that the dialysate possessed a strong hydrolyzing effect on proteins. While it was not as active as the original ferment solution, the residue remaining in the bag was found to be practically inactive. The principal method used by Herzfeld in his experiments and by which he determined the rate of protein hydrolysis was the colorimetric or spectrophotometric comparison of the color developed by an evaporation of the solution to be examined with Ruhemann's reagent (ninhydrin) and compared with a glycine solution of known strength. There are two objections to Herzfeld's theory that the action of trypsin is due to the presence of protein cleavage products: (1) Ninhydrin was found to be only to some extent specific for amino-acids, as allantoin[3] and other substances[4] give a coloration with this reagent under certain conditions. (2) The work of Holderer,[5] and Funk and Niemann[6] shows that trypsin does not filter through a Chamberland filter in

[1] Herzfeld, E., *Biochem. Z.*, 1915, lxviii, 402.
[2] Herzfeld, *Biochem. Z.*, 1915, lxx, 262.
[3] Funk, C., *Biochem. J.*, 1913, vii, 211.
[4] Neuberg, C., *Biochem. Z.*, 1913, lvi, 495.
[5] Holderer, M., *Compt. rend. Acad.*, 1910, cl, 285.
[6] Funk, C., and Niemann, A., *Z. physiol. Chem.*, 1910, lxviii, 263.

neutral solution, and, therefore, the action of trypsin cannot be due to the presence in it of amino-acids, as under these conditions the latter would pass through the filter readily. It has since been found by the author[7] that trypsin on filtration in neutral solution is so strongly adsorbed or inactivated that even breaking the porous porcelain filter to a fine powder and extracting it with weak acid or alkali will not regenerate the power of the ferment. Recovery of the ferment would be expected if the active part of trypsin were composed of cleavage products of proteins.

Under these circumstances it was found advisable to test Herzfeld's results by a different method than that used in his work, and we decided to use Van Slyke's method for the determination of amino nitrogen, and, in some instances, also the micro Kjeldahl method as recommended by Pregl.[8] We may mention here that the results we have obtained do not support Herzfeld's view that the action of trypsin is mainly due to the presence of amino-acids, and the difference in the results obtained may be due either to the fact that a modified technique was used by us or that the intensified color obtained with ninhydrin was not due to an actual increase in amino-acids.

Dialysis of Trypsin.

Herzfeld in his experiments used commercial trypsin preparations of unspecified origin, and he states that trypsins with a high nitrogen content gave the best results on dialysis. The nitrogen content was not quite so high in the trypsin preparations we used as in those of this author. Solutions of different strengths were dialyzed in collodion membranes (instead of the parchment bags of Schleicher and Schull) against distilled water, the liquid being kept covered in all cases with toluene (numerous controls have entirely excluded any bacterial action in our experiments). The method employed was as follows: The original ferment solution, the dialysate, and the residue were tested separately as to their action on freshly prepared fibrin or egg albumin, then the protein and the solution to be examined were placed in collodion bags with distilled water outside, both liquids covered with tolu-

[7] Unpublished work.

[8] Pregl, F., *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1912, v, 1344.

ene, and incubated at 37° for 24 hours. The dialysate was then poured off, washed into a fractionation flask, and evaporated *in vacuo*. The residue was washed into a measuring flask and an aliquot part analyzed for amino nitrogen and in some cases for total nitrogen.

Experiment 1.—A 4 per cent solution of Fairchild's trypsin (11.27 per cent N) was made and 5 cc. of it were placed in a bag with 50 cc. of water outside, while another 5 cc. of the same solution were put directly into 50 cc. of water. Both beakers were incubated for 24 hours. We have used stronger solutions of the ferment than Herzfeld in order to magnify the ultimate results. After incubation the residue in the bag was again diluted to 50 cc. Amino nitrogen determinations were made on the three solutions: (1) the original ferment, (2) the dialysate, and (3) the residue.

1. Original ferment gave 0.0064 gm. NH_2 N.
2. Dialysate " 0.0051 " "
3. Residue " 0.0012 " "

From these analyses we can see that a large part of the amino-acids originally present in the ferment solution dialyzed out.

	NH_2 N found: gm.
5 cc. Solution 1 in a bag*	0.000075
5 " " 1 " " " and 0.5 gm. egg albumin	0.00011
5 " " 1 " " " " 0.5 " fibrin	0.00016
0.5 gm. fibrin and 5 cc. water in a bag	0
0.5 " egg albumin and 5 cc. water in a bag	0
5 cc. dialysate in a bag	0.00012
5 " " " " " and 0.5 gm. egg albumin	0.000088
5 " " " " " " 0.5 " fibrin	0.000101
5 cc. residue in a bag	0.000051
5 " " " " " and 0.5 gm. egg albumin	0.000057
5 " " " " " " 0.5 " fibrin	0.000037

* 40 cc. of water outside, in each case.

Experiment 2.—In this experiment a 10 per cent solution of the same trypsin preparation was used and fibrin as a protein substrate.

1. Original ferment gave 0.0246 gm. NH_2 N.
2. Dialysate " 0.0224 " "
3. Residue " 0.00556 " "

	NH_2 N found: gm.
5 cc. original ferment in a bag*	0.0059
5 " " " " " " and 0.5 gm. fibrin	0.0182
0.5 gm. fibrin and 5 cc. water inside	0.0010
5 cc. dialysate in a bag	0.0087
5 " " " " " and 0.5 gm. fibrin	0.0084
5 cc. residue in a bag	0.0020
5 " " " " " and 0.5 gm. fibrin	0.0054

* 40 cc. of water outside, in each case.

In the above experiment with Fairchild's trypsin, which, upon repetition gave the same results, we find a diminution of proteoclastic action both in the dialysate and the residue as compared with the original ferment solution. Furthermore the dialysate does not show any proteoclastic effect on the protein, but on the contrary, a diminution of amino nitrogen.

Experiment 3.—In this experiment trypsin from Armour and Company (12.19 per cent N) was used and the proteolysis was determined both by the amino nitrogen and by total nitrogen determinations using the micro Kjeldahl method. A 10 per cent solution of the ferment was employed.

1. Original ferment gave 0.026 gm. NH_2 N.
2. Dialysate " 0.0145 " "

	NH_2 N. gm.	Total N. gm.
5 cc. original trypsin in a bag*	0.0020	0.00435
5 " " " " " " and 0.5 gm. egg albumin	0.0037	0.0067
0.5 gm. fibrin in a bag	0.00038	0.0005
5 cc. dialysate in a bag	0.0027	0.00435
5 " " " " " and 0.5 gm. egg albumin	0.0027	0.0034
5 cc. residue in a bag	0.00088	0.0011
5 " " " " " and 0.5 gm. egg albumin	0.00145	0.0031

* 40 cc. of water outside, in each case.

The dialysate, although very much richer in amino nitrogen than the residue, had no hydrolyzing effect on the protein, and the residue was very much more active than the dialysate. The dialysate showed a diminution of amino nitrogen and total nitrogen in the outside fluid, as in all our experiments where a dialyzing membrane was used. Herzfeld considers also a possible synthetic action of the ferment, or amino-acid. We prefer to explain this phenomenon for the present as a mere adsorption of the cleavage products by the membrane, or by the protein in the dialyzing bag.

Experiment 4.—Pancreatine from Parke, Davis and Company in 10 per cent solution was used (11.92 per cent N), the insoluble part being filtered off.

1. Original ferment gave 0.0192 gm. NH_2 N.
2. Dialysate " 0.0128
3. Residue " 0.00286

	NH_2 N. *gm.*	Total N. *gm.*
5 cc. original trypsin in a bag*	0.00254	0.0043
5 " " " " " " and 0.5 gm. fibrin	0.00996	0.0185
0.5 gm. fibrin and 5 cc. water in a bag	0.00047	0.00125
5 cc. dialysate in a bag	0.00206	0.00345
5 " " " " " and 0.5 gm. fibrin	0.00194	0.00340
5 cc. residue in a bag	0.00044	0.00095
5 " " " " " and 0.5 gm. fibrin	0.00269	0.0053

* 40 cc. of water outside, in each case.

After having found that the dialyzing portion of the trypsin had, in our experiments, no proteolytic action on proteins we repeated some of Herzfeld's experiments on the proteoclastic action of amino-acids[9] and peptides on proteins.

Action of Protein Cleavage Products on Proteins.

Herzfeld has shown, by means of his colorimetric ninhydrin method, that amino-acids and peptides have a distinct proteolytic

[9] We are indebted for certain of these preparations to Dr. W. G. Lyle and Dr. P. A. Levene.

action on certain proteins, the aliphatic amino-acids being more effective than the aromatic ones. In one case he performed a direct experiment, allowing glycine to act on fibrin. On evaporation of the dialysates, and drying to constant weight, he found that the residue obtained from the action of glycine on fibrin weighed more than the residues obtained in the control experiment. We repeated this experiment using the amino nitrogen determination as an index of hydrolysis, but, as glycine does not react in the usual way with nitrous acid and gives more than the theoretical amount of gas, another experiment was done for the total nitrogen estimation.

Glycine and Fibrin.

A 2 per cent solution of glycine was used, which was added directly to the fibrin in a bag.

	NH_2 N. gm.
5 cc. glycine solution	0.0184
5 " " " and 0.5 gm. fibrin	0.01856
0.5 gm. fibrin	0.00018

We found no increase of amino nitrogen. In another experiment a 4.9 per cent solution of glycine was used without dialysis and the solutions covered with toluene were left in the incubator for 6 days.

	NH_2 N. gm.	Total N. gm.
5 cc. glycine in 40 cc. water	0.0495	0.0356
5 " " and 0.5 gm. fibrin	0.0482	0.0461
0.5 gm. fibrin	0.00058	0.0014

In this experiment again we find no increase in amino nitrogen, but, in confirmation of Herzfeld's result, an increase in total nitrogen. This at present cannot be explained, although it was obtained by us in subsequent experiments, but not to such a marked degree as in the case of glycine.

Action of Other Amino-Acids and Peptides on Protein.

	NH_2 N. gm.	Total N. gm.
5 cc. glycyl-glycine (0.8 per cent)*	0.004	
0.5 gm. fibrin	0.0045	
5 cc. glycyl-glycine and 0.5 gm. fibrin	0.0004	
5 cc. *l*-leucine (1.9 per cent)	0.0106	0.0108
5 " " (1.9 " "), and 0.5 gm. fibrin	0.0106	0.01175†
10 cc. *dl*-leucine (0.93 per cent)‡	0.00925	0.0095
10 " " (0.93 " "), and 0.5 gm. fibrin	0.00925	0.0112
0.5 gm. fibrin	0.000287	0.0027
5 cc. *l*-alanyl-*d*-alanine (0.8 per cent)	0.0012	
5 " " and 0.5 gm. egg albumin	0.0014	
0.5 gm. egg albumin	0.0003	
Completely hydrolyzed casein, acid quantitatively removed, 25 gm. in 500 cc., 5 cc. taken	0.01457	0.02137
The same and 0.5 gm. fibrin	0.01505	0.0225
0.5 gm. fibrin	0.000189	0.00025
5 cc. trypsin dialysate (Armour)	0.001806	0.00375
5 " " " " and 5 cc. of the above casein hydrolysate	0.01646	0.02514

* 40 cc. of water in each case.
† Left for 5 days.
‡ 35 cc. of water in this case.

This experiment was performed in order to test whether the dialysate possesses synthetical action. This phenomenon could not be demonstrated as the amounts calculated in the last experiment were 0.01637 NH_2 N and 0.02512 total N, which closely corresponded to our results. We have also attempted testing the synthetic action of the ferment itself, or its dialysate, by the optical method and also by direct incubation of amino-acid with the ferment. None of the experiments were successful, nor were we able to detect any hydrolyzing properties of the cleavage products in their action on proteins.

SUMMARY.

Trypsin, when subjected to dialysis in collodion bags, loses a large part of its amino nitrogen, corresponding to the passage of amino-acids into the dialysate. The dialysate was found to be without hydrolyzing effect on proteins, whereas, in most cases the residue was found to have distinct proteoclastic properties. The dialysis of trypsin was followed by a marked inactivation of the ferment. The diminution of amino nitrogen, where the dialysate was allowed to act upon protein, is perhaps due to the adsorption of a certain quantity of the cleavage products by the membrane, or by the protein substrate, and is apparently not due to synthetic action. The amino-acids and peptides used had no hydrolyzing effect on proteins, as an increase of amino nitrogen was never found. Occasionally, as in the case of glycine, an increase in total nitrogen was found, for which we can offer no explanation.

THE INFLUENCE OF ETHYL ALCOHOL AND GLYCEROL UPON THE RATE OF SOLUTION OF CASEIN BY SODIUM HYDROXIDE.

By T. BRAILSFORD ROBERTSON and K. MIYAKE.

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In previous articles[1] we have shown that the rate of solution of casein in an alkaline solvent is determined by the velocity with which the casein particles are penetrated and wetted by the solvent, the relationship between the time of exposure to the solvent (t) and the amount of casein dissolved (x) being expressed by the formula $x = Kt^m$, in which K and m are constants which vary with the nature of the solvent. The product $K \times m$ we have called the coefficient of penetration, and we have shown that the addition of alkali or alkaline earth chlorides to an alkaline solution employed as solvent decreases the value of the coefficient of penetration and, consequently, the rate of solution of the casein.

It appeared of special interest in this connection to study the effect upon the rate of solution of casein in alkaline solvents of organic substances which are known to exert a marked effect upon surface tension. Alcohol and glycerol are known to reduce the tension of a solid-water interface and the addition of these substances to the alkaline solvent should evidently, if the above outlined theory correctly represents the facts, retard the rate of penetration and, consequently, the rate of solution of the casein. While, as will be seen, our results are in harmony with the requirements of the theory, certain anomalies were encountered in the behavior of the solvents containing

[1] Robertson, T. B., *J. Phys. Chem.*, 1910, xiv, 377; *Arch. ges. Physiol.*, 1913, clii, 524. Robertson, T. B., and Miyake, K., *J. Biol. Chem.*, 1916, xxv, 351.

alcohol, anomalies which are attributable to the fact that alcohol is a protein coagulant, or dehydrating agent.

The casein employed in these experiments was the same as that used in our previously reported investigations and the experimental technique was also the same. As a standard alkaline solvent we employed 0.016 N sodium hydroxide containing varying concentrations of alcohol or glycerol. The volume of solvent employed was always 100 cc.; 5 gm. of casein were dropped into the solvent and the mixture was stirred at an approximately constant rate. Samples of the mixture were withdrawn from time to time, rapidly filtered, and the concentration of casein contained in the filtrate was determined by the refractometric method.

Tables I to XIV embody the results obtained with the solvents containing alcohol, and these results are also depicted graphically in Figs. 1 and 2. It will be seen that the addition of alcohol markedly decreases the rate of solution of casein by dilute sodium hydroxide. In the mixtures containing over 4 M and less than 8 M alcohol, however, a curious phenomenon is observed. It will be seen, on consulting Tables VII to XI, that in these mixtures a partial reprecipitation of the casein initially dissolved occurs, so that after 2 hours of stirring in the mixtures containing from 4.5 M to 6 M alcohol (Tables VII to X) the amount of casein in solution is actually less than it is after $\frac{1}{2}$ hour of stirring.

TABLES.

t	i	n	Casein in 100 cc. of solvent.		Δ
			Observed.	Calculated.	
I. Solvent: 0.016 N NaOH. i = 68.05. n_1 = 1.329250. Temperature: 23.5–24°. K = 2.371. m = 0.151.					
			gm.	*gm.*	
5	67.07	1.333714	2.98	3.02	+0.04
10	66.57	1.334497	3.45	3.36	−0.09
30	66.45	1.335445	4.08	3.96	−0.08
60	66.38	1.335990	4.43	4.40	−0.03
120	66.32	1.336470	4.75	4.91	+0.16
					ΣΔ = ±0.00

t	i	n	Casein in 100 cc. of solvent.		Δ
			Observed.	Calculated.	
II. Solvent: 0.016 N NaOH + 0.5 M C_2H_5OH. i = 67.39. n_1 = 1.331237. Temperature: 23-23.5°. K = 2.214. m = 0.148.					
			gm.	*gm.*	
5	66.45	1.335445	2.77	2.82	+0.05
10	66.37	1.336070	3.18	3.11	−0.07
30	66.26	1.336950	3.76	3.66	−0.10
60	66.20	1.337430	4.08	4.06	−0.02
120	66.15	1.337830	4.34	4.50	+0.16
					$\Sigma\Delta$ = +0.02
III. Solvent: 0.016 N NaOH + 1 M C_2H_5OH. i = 67.26. n_1 = 1.332238. Temperature: 23-23.5°. K = 2.065. m = 0.149.					
5	66.36	1.336150	2.57	2.62	+0.05
10	66.29	1.336710	2.94	2.91	−0.03
30	66.18	1.337590	3.52	3.43	−0.09
60	66.12	1.338070	3.84	3.80	−0.04
120	66.07	1.338470	4.10	4.21	+0.11
					$\Sigma\Delta$ = ±0.00
IV. Solvent: 0.016 N NaOH + 2 M C_2H_5OH. i = 66.45. n_1 = 1.335445. Temperature: 23°. K = 1.862. m = 0.149.					
5	65.59	1.339111	2.41	2.37	−0.04
10	65.56	1.339354	2.70	2.62	−0.08
30	65.47	1.340083	3.05	3.09	+0.04
60	65.42	1.340488	3.32	3.43	+0.11
120	65.38	1.341284	3.84	3.80	−0.04
					$\Sigma\Delta$ = +0.01
V. Solvent: 0.016 N NaOH + 3 M C_2H_5OH. i = 66.12. n_1 = 1.338070. Temperature: 23°. K = 1.631. m = 0.147.					
5	65.33	1.341224	2.08	2.07	−0.01
10	65.28	1.341634	2.34	2.29	−0.05
30	65.21	1.342208	2.72	2.69	−0.03
60	65.18	1.342454	2.90	2.98	+0.08
120	65.17	1.342536	2.94		
					$\Sigma\Delta$ = −0.01

t	i	n	Casein in 100 cc. of solvent.		Δ
			Observed.	Calculated.	
VI. Solvent: 0.016 N NaOH + 4 M C_2H_5OH. i = 65.22. n_1 = 1.342126. Temperature: 23°. K = 1.540. m = 0.145.					
			gm.	*gm.*	
5	64.46	1.345106	1.96	1.94	−0.02
10	64.43	1.345358	2.13	2.15	+0.02
30	64.40	1.345610	2.29		
60	64.38	1.345776	2.40		
120	64.38	1.345776	2.40		
					$\Sigma\Delta$ = ±0.00
VII. Solvent: 0.016 N NaOH + 4.5 M C_2H_5OH. i = 65.03 n_1 = 1.343691. Temperature: 23–23.5°.					
5	64.28	1.346608	1.92		
10	64.25	1.346860	2.08		
30	64.21	1.347196	2.31		
60	64.22	1.347112	2.25		
120	64.24	1.346944	2.14		
VIII. Solvent: 0.016 N NaOH + 5 M C_2H_5OH. i = 64.42. n_1= 1.345442. Temperature: 22–22.5°.					
5	64.09	1.358215	1.82		
10	64.08	1.348280	1.87		
30	64.08	1.348280	1.87		
60	64.11	1.348045	1.71		
120	64.19	1.347365	1.40		
IX. Solvent: 0.016 N NaOH + 5.5 M C_2H_5OH. i = 64.26. n_1 = 1.346776. Temperature: 23–23.5°.					
5	63.54	1.349490	1.79		
10	63.52	1.349660	1.90		
30	63.51	1.349745	1.95		
60	63.53	1.349575	1.84		
120	63.59	1.349065	1.51		

t	i	n	Casein in 100 cc. of solvent.		Δ
			Observed.	Calculated.	
X. Solvent: 0.016 N NaOH + 6 M C_2H_5OH. i = 64.07. n_1 = 1.348385. Temperature: 23–23.5°.					
			gm.	*gm.*	
5	63.37	1.350948	1.69		
10	63.35	1.351120	1.80		
30	63.32	1.351378	1.97		
60	63.30	1.351550	2.08		
120	63.32	1.351378	1.97		
XI. Solvent: 0.016 N NaOH + 7 M C_2H_5OH. i = 63.20. n_1 = 1.351378. Temperature: 22–22.5°.					
5	63.03	1.353879	1.65		
10	63.00	1.354140	1.82		
30	62.58	1.354304	1.93		
60	62.55	1.354575	2.10		
120	62.56	1.354488	2.05		
XII. Solvent: 0.016 N NaOH + 8 M C_2H_5OH. i = 63.13. n_1 = 1.353012. Temperature: 23–23.5°. K = 1.333. m = 0.139.					
5	62.44	1.355532	1.66	1.66	±0.00
10	62.41	1.355793	1.83	1.83	±0.00
30	62.37	1.356144	2.06		
60	62.36	1.356232	2.12		
120	62.355	1.356276	2.15		
					ΣΔ = ±0.00
XIII. Solvent: 0.016 N NaOH + 9 M C_2H_5OH. i = 62.47. n_1 = 1.355271. Temperature: 23°. K = 1.281. m = 0.141.					
5	62.18	1.357816	1.67	1.60	−0.07
10	62.16	1.357992	1.79	1.77	−0.02
30	62.10	1.358520	2.14	2.07	−0.07
60	62.08	1.358692	2.25	2.28	+0.03
120	62.06	1.358872	2.37	2.52	+0.15
					ΣΔ = +0.02
XIV. Solvent: 0.016 N NaOH + 10 M C_2H_5OH. i = 62.28. n_1 = 1.356936. Temperature: 23°. K = 1.149. m = 0.144.					
5	62.04	1.359048	1.39	1.42	+0.03
10	62.01	1.359312	1.56	1.60	+0.04
30	61.54	1.359934	1.97	1.88	−0.09
60	61.51	1.360201	2.15	2.07	−0.08
120	61.50	1.360290	2.21	2.29	+0.08
					ΣΔ = −0.02

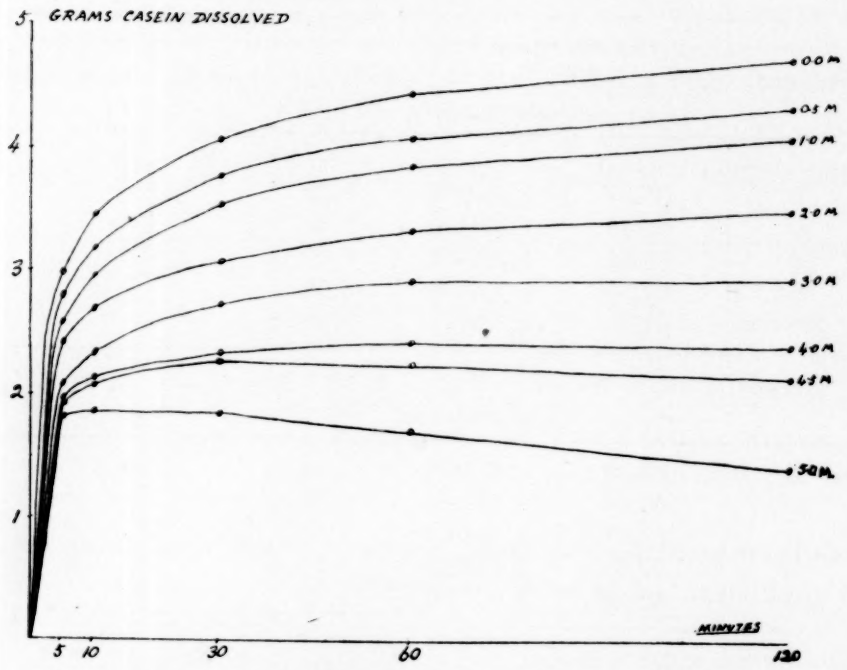

FIG. 1. The influence of ethyl alcohol upon the rate of solution of casein by 0.016 N sodium hydroxide.

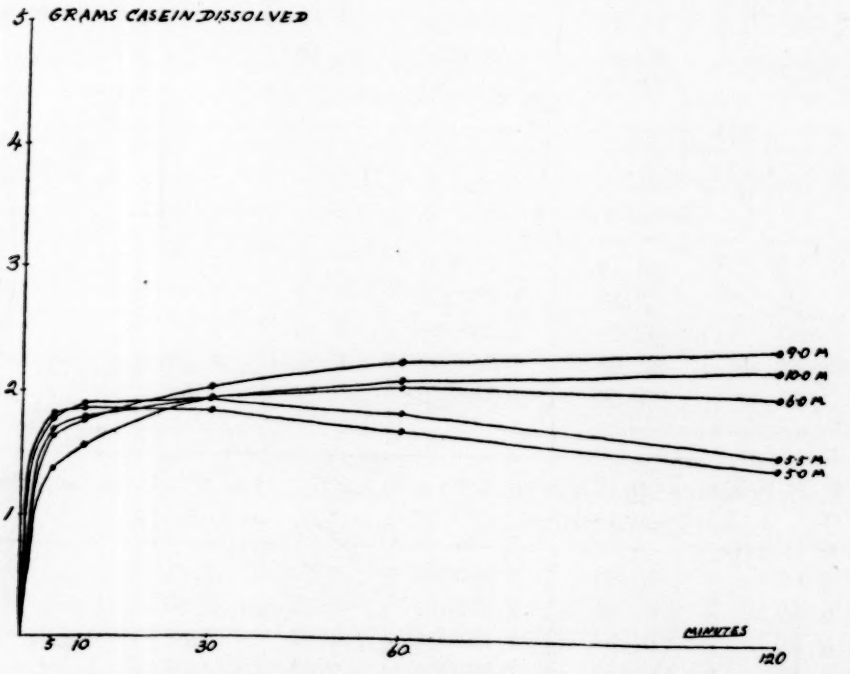

FIG. 2. The influence of ethyl alcohol upon the rate of solution of casein by 0.016 N sodium hydroxide.

It is evident that in mixtures which contain between 4 M and 8 M alcohol the formula $x = Kt^m$ does not apply to the rate of solution of the casein, for this formula implies a continuous irreversible process, while in the presence of the above mentioned concentrations of alcohol the process of solution is evidently partially reversible.

Above and below this critical zone of concentrations the relation between the time of stirring and the concentration of casein dissolved is adequately expressed by the formula cited, as will be evident on comparing calculated and observed values of x in Tables I to VI and XII to XIV inclusive, the deviations of the calculated from the observed values rarely being greater than the possible error in the refractometric determination of x.

On investigating the effect of alcohol upon the magnitude of the coefficient of penetration (Table XV and Fig. 3) it is evi-

XV. C_2H_5OH.

I. $K_1m_1 = +0.358$. $\alpha = +0.04945$. $\beta = -0.003395$.

II. $K_1m_1 = 0.185$. $\alpha = -0.001585$. $\beta = +0.00586$.

Concentration.	K	m	Km. Found.	Km. Calculated. I.	Km. Calculated. II.	Difference. I.	Difference. II.
			gm.	gm.	gm.	gm.	gm.
0.0	2.371	0.151	0.358	0.358		±0.000	
0.5	2.214	0.148	0.328	0.334		+0.006	
1.0	2.065	0.149	0.308	0.313		+0.005	
2.0	1.862	0.149	0.277	0.273	−0.036	−0.004	
3.0	1.631	0.147	0.240	0.240	0.031	±0.000	
4.0	1.540	0.145	0.223	0.225	0.085	+0.003	
4.5				0.204	0.108		
5.0				0.199	0.127		
5.5				0.189	0.144		
6.0				0.184	0.158		
7.0				0.178	0.178		
8.0	1.333	0.139	0.185	0.180	0.185		±0.000
9.0	1.281	0.141	0.181	0.188	0.181		±0.000
10.0	1.149	0.144	0.165	0.203	0.165		±0.000
11.0				0.225	0.137		
12.0				0.253	0.098		
13.0				0.289	0.046		
14.0				0.331	−0.016		

dent that in the zone of concentrations lying between 4 M and 8 M alcohol the character of the relationship between the concentration of alcohol and the magnitude of the coefficient of penetration is undergoing a transition. For concentrations of alcohol lying below 4.5 M the coefficient of penetration decreases with negative acceleration as the concentration of alcohol increases. In mixtures containing over 7 M alcohol, however, the coefficient of penetration decreases with positive acceleration as the concentration of alcohol increases. In mixtures containing concentrations of alcohol lying between 4.5 M and 7 M inclusive, the character of the relationship is indeterminate.

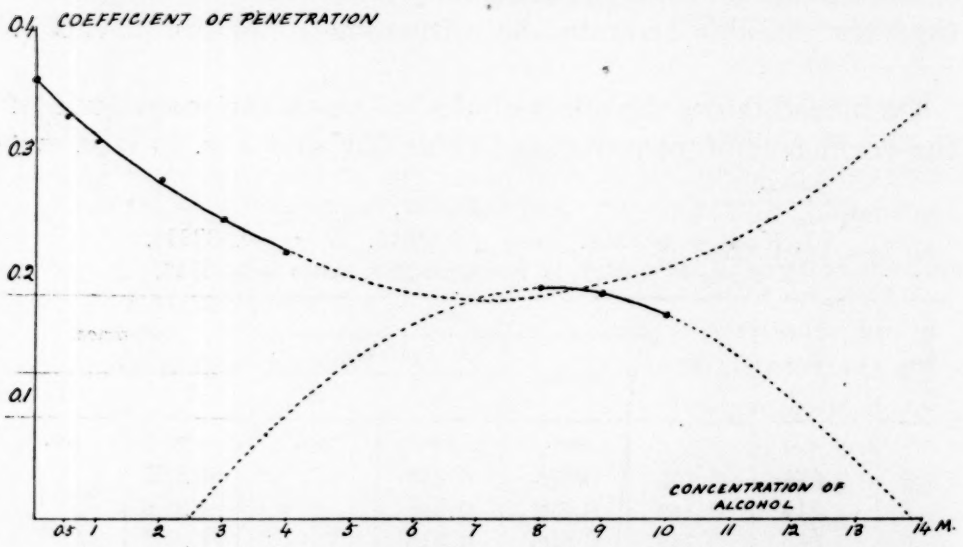

FIG. 3. The influence of ethyl alcohol upon the coefficient of penetration. The broken lines indicate the calculated continuations of the continuous lines drawn through the experimentally determined points.

Applying the interpolation equation employed in our previous article, $K_1m_1 - Km = \alpha c + \beta c^2$ where $K_1m_1 - Km$ represents the decrease in the coefficient of penetration, c represents the concentration of alcohol, and α and β are constants, we find that for concentrations of alcohol lying below 4.5 M ($K_1m_1 = 0.358$) the equation becomes:

$$0.358 - Km = +0.0495\,c - 0.00340\,c^2$$

while for concentrations lying above 8 M ($K_1m_1 = 0.185$) the equation becomes:

$$0.185 - Km = -0.00159\ (c-8) + 0.00586\ (c-8)^2$$

which implies that at concentrations of alcohol lying below 8.27 M the rate of solution increases with increasing concentration of alcohol.

The only explanation of these curious phenomena which offers itself lies in the fact that alcohol is a coagulant of proteins and therefore brings about dehydration and consequent polymerization of protein molecules.[2] At low concentrations of alcohol we have to deal with the rate of solution of "single" molecules of sodium caseinate, at higher concentrations with the rate of solution of polymerized molecules which we may, for the sake of brevity, term "double" molecules, while at intermediate concentrations we have to deal with a rate of solution which is compounded of the separate rates of solution of the two types of molecules. With increasing concentration of alcohol the rate of solution of the single molecules is progressively diminished, but that of the double molecules is increased, owing to the fact that the proportion of double to single molecules coming off from the internal surfaces of the casein particles is being increased sufficiently rapidly to more than compensate for the retardation of the total rate of solution by the alcohol. At 8 M concentration so large a proportion of the molecules coming off are of the double type that thereafter the retarding effect of increasing concentration of alcohol upon the total rate of solution (penetration) more than compensates for any further increase in the proportion of double molecules.

At intermediate concentrations we have seen that the quantity of casein dissolved at first increases and thereafter diminishes with time, casein which is initially dissolved being later reprecipitated. This can only be interpreted by supposing that the condition of the bulk of the solution outside the casein particles differs, at any rate for some portion of the time occupied in solution, from the condition of that portion of the solvent which has actually penetrated the protein particles. We know that any substance such as alcohol, which reduces the tension of a solid-water interface, tends to become concentrated at such

[2] Robertson, Die physikalische Chemie der Proteine, Dresden, 1912.

an interface.[3] Hence at the surfaces of the casein particles the concentration of alcohol will be greater than in the bulk of the fluid. Suppose that the concentration of alcohol in the bulk of the fluid be such that at equilibrium the proportion of double molecules is 50 per cent. If, now, the concentration of alcohol at the surface of the casein particles be such as to lead to the formation of 60 per cent of double molecules, then out of every thirty molecules coming off from the interior surfaces of the casein particles eighteen will be of the double type and only twelve of the single type. As these come out into the bulk of the liquid, equilibrium will tend to be reestablished, resulting in the formation of sixteen double and sixteen single molecules, with an increase in the total number of molecules to thirty-two. This equilibrium may be supposed to be slowly established, and meanwhile the rate of solution of the casein has fallen very low; that is, the fall in concentration or concentration gradient from within the casein particles to the bulk of the fluid outside is very small. By this time, however, as a result of depolymerization, the molecular concentration of casein in the outer fluid has actually become greater than that in the saturated solution which fills the interior spaces of the casein particles. Thus the concentration gradient has become negative, and as the more concentrated casein solution diffuses back into the less concentrated but nevertheless saturated solution filling the interspaces of the sponge-like particles of casein, the excess of casein must be reprecipitated. When the concentration of alcohol is sufficiently great, so that the molecular condition of the casein is practically the same within and without the particles, a negative concentration gradient can never arise and the curve of solution reassumes the normal form, representing now, however, the curve of solution of double molecules instead of single molecules as at lower concentrations of alcohol.

It may here be pointed out that the observed results cannot be interpreted as the result of errors in the determination of casein due to abnormal refractivity in alcohol-water mixtures, since the refractivity of casein dissolved in alcohol-water mix-

[3] Thomson, J. J., Application of Dynamics to Physics and Chemistry, London, 1888, 190.

tures of the proportions employed is normal.[4] On the other hand, it has been directly shown by electrolytic transference measurements that the weight of the casein particle carrying a unit charge is progressively increased by the addition of increasing concentrations of alcohol to the solution.[5] We are justified in concluding from the above data, therefore, that at concentrations of alcohol lying below 4.5 M casein molecules in the presence of 0.016 N NaOH are practically all of the single type, while at concentrations lying above 7.0 M they are practically all of a polymerized type. At intermediate concentrations of alcohol the proportion of polymerized to single molecules continuously increases with increasing concentration of alcohol.

This interpretation of the above facts finds further confirmation in the effect of glycerol upon the rate of solution of casein in dilute alkali. In glycerol we have a substance which, like alcohol, reduces the tension of a solid-water interface but which, unlike alcohol, does not coagulate (polymerize) proteins in solution. Accordingly we find that glycerol (Tables XVI to XX) decreases the rate of solution of casein progressively as its concentration increases. No sign of reprecipitation of dissolved casein is observed in any of the mixtures. The penetration formula $x = Kt^m$ applies to the rate of solution in all of the mixtures employed and the value of the coefficient of penetration progressively decreases in a smooth curve with decreasing acceleration as the concentration of glycerol increases (Fig. 4). For all the concentrations of glycerol employed, the relationship of the coefficient of penetration to the concentration of glycerol may be expressed by the equation:

$$K_1 m_1 - Km = 0.0525\, c - 0.00243\, c^2.$$

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[4] Robertson, *J. Biol. Chem.*, 1910, viii, 507.

[5] Robertson, *J. Phys. Chem.*, 1911, xv, 387.

t	i	n	Casein in 100 cc. of solvent.		Δ
			Observed.	Calculated.	
XVI. Solvent: 0.016 N NaOH + 1 M $C_3H_5(OH)_3$. i = 65.55. n_1 = 1.339435. Temperature: 23-23.5°. K = 1.779. m = 0.173.					
			gm.	*gm.*	
5	65.13	1.342964	2.32	2.35	+0.03
10	65.06	1.343442	2.64	2.65	+0.01
30	64.55	1.344355	3.24	3.20	−0.04
60	64.48	1.344938	3.62	3.61	+0.01
120	64.40	1.345610	4.06	4.07	−0.01
					$\Sigma\Delta$ = ±0.00
XVII. Solvent: 0.016 N NaOH + 3 M $C_3H_5(OH)_3$. i = 62.26. n_1 = 1.357112. Temperature: 24°. K = 1.217. m = 0.173.					
5	61.58	1.359578	1.62	1.61	−0.01
10	61.55	1.359845	1.80	1.81	+0.01
30	61.48	1.360468	2.21	2.19	−0.02
60	61.44	1.360824	2.44	2.47	+0.03
120	61.38	1.361360	2.79	2.79	±0.00
					$\Sigma\Delta$ = +0.01
XVIII. Solvent: 0.016 N NaOH + 5 M $C_3H_5(OH)_3$. i = 58.08. n_1 = 1.380688. Temperature: 23-23.5°. K = 0.9226. m = 0.170.					
5	57.47	1.382675	1.31	1.21	−0.10
10	57.46	1.382770	1.37	1.37	±0.00
30	57.44	1.382960	1.49	1.65	+0.16
60	57.39	1.383435	1.81	1.85	+0.04
120	57.33	1.384005	2.18	2.08	−0.10
					$\Sigma\Delta$ = ±0.00
XIX. Solvent: 0.016 N NaOH + 7 M $C_3H_5(OH)_3$. i = 54.25. n_1 = 1.402205. Temperature: 24°. K = 0.6919. m = 0.173.					
5	54.09	1.403778	1.03	0.91	−0.12
10	54.08	1.403876	1.10	1.03	−0.07
30	54.07	1.403974	1.16	1.25	+0.09
60	54.05	1.404170	1.29	1.41	+0.13
120	54.00	1.404660	1.62	1.58	−0.04
					$\Sigma\Delta$ = −0.01

t	i	n	Casein in 100 cc. of solvent.		Δ
			Observed.	Calculated.	
XX. Solvent: 0.016 N NaOH + 10 M $C_3H_5(OH)_3$. i = 50.35. n_1 = 1.42505. Temperature: 24°. K = 0.4216. m = 0.178.					
			gm.	*gm.*	
5	50.27	1.42585	0.53	0.56	+0.03
10	50.25	1.42605	0.66	0.64	−0.02
30	50.23	1.42625	0.79	0.77	−0.02
60	50.22	1.42635	0.89	0.87	−0.02
120	50.20	1.42655	0.99	0.99	±0.00
					$\Sigma\Delta$ = −0.03

XXI. $C_3H_5(OH)_3$. K_1m_1 = +0.358. α = +0.05251. β = −0.00243.

Concentration.	K	m	Km.		Difference.
			Found.	Calculated.	
			gm.	*gm.*	*gm.*
0	2.3710	0.151	0.358	0.358	±0.000
1	1.7790	0.173	0.308	0.308	±0.000
3	1.2170	0.173	0.211	0.222	+0.011
5	0.9226	0.170	0.157	0.156	−0.001
7	0.6919	0.173	0.120	0.110	−0.010
10	0.4216	0.178	0.075	0.076	+0.001

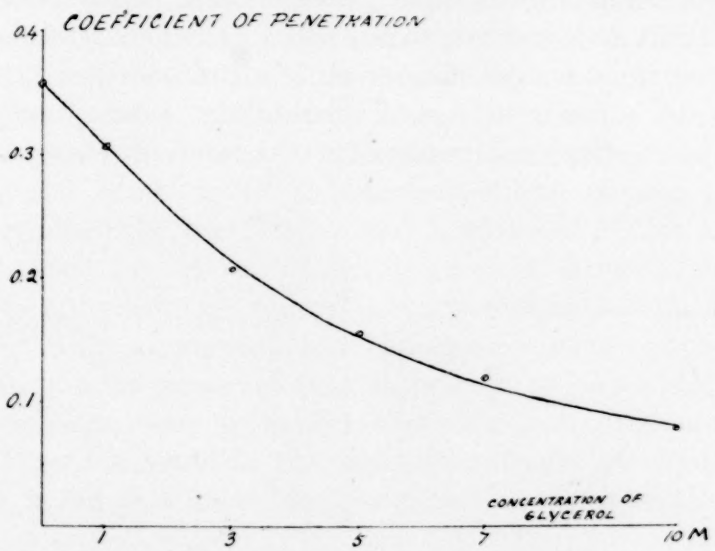

FIG. 4. The influence of glycerol upon the coefficient of penetration.

SUMMARY.

1. Alcohol and glycerol, both of which reduce the tension of a solid-water interface, also retard the penetration of casein particles by 0.016 N NaOH.

2. The penetration formula $x = Kt^m$ expresses the relationship between the quantity of casein dissolved and the time of stirring in all the glycerol-water mixtures studied and in alcohol-water mixtures containing less than 4.5 M or more than 7 M alcohol. The formula does not apply to the rate of solution of casein in 0.016 N NaOH containing between 4 M and 8 M alcohol. In these mixtures the initial solution of casein is succeeded, after prolonged stirring, by partial reprecipitation.

3. In concentrations lying below 4.5 M the coefficient of penetration decreases with negative acceleration as the concentration of alcohol increases. In concentrations lying above 7 M the coefficient of penetration decreases with positive acceleration as the concentration of alcohol increases. In mixtures containing concentrations of alcohol lying between 4.5 M and 7 M, inclusive, the character of the relationship of the magnitude of the coefficient of penetration to the concentration of alcohol is indeterminate.

4. It is inferred that in concentrations of alcohol lying below 4.5 M casein molecules in the presence of 0.016 N NaOH are practically all of the single type, while at concentrations lying above 7.0 M they are practically all of a polymerized type. In intermediate concentrations of alcohol the proportion of the polymerized to the single molecules progressively increases with increasing concentration of alcohol.

CHONDROSAMINE.

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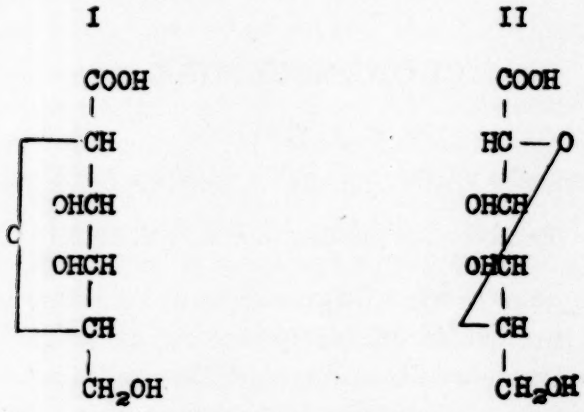
By the previous work of Levene and La Forge[1] there were established a number of facts concerning chondrosamine. One of the principal ones was that on oxidation with nitric acid chondrosaminic acid was transformed into an inactive anhydrotetrahydroxyadipic acid. This fact alone suffices to limit the possible configurations of chondrosamine to that of a lyxohexosamine or of a ribohexosamine. Two other facts swung the weight of the argument in favor of the second hypothesis. They were, first, the similarity of the osazones obtained from altrose and from chondrosamine. The similarity was found in their melting points and in their optical activity. The other fact was that two hexosaminic acids, lyxohexosaminic and chondrosaminic, very different in their physical properties, led to formation of inactive anhydrotetrahydroxyadipic acids. Accepting that all hexosaminic acids showed the same behavior in the sense of Walden's inversion, one was forced to the conclusion that chondrosamine is not a lyxohexosamine, and hence is a ribosimine. The conclusion seemed perfectly logical. Subsequent development of the work has caused some doubt in the correctness of the conclusion.

Chronologically our faith in the correctness of this assumption was shaken first by the observations on the optical rotation of the two anhydropentoxycaproic acids obtained from chondrosamine. On the assumption that chondrosamine is a ribohexosamine it must also be assumed that it belongs to the *l* series. Were this true, one was to expect that the anhydropentoxycaproic acid (I) which leads to the inactive anhydrotetrahydroxyadipic acid is to have a lower dextrorotation or a higher levorotation

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 123; 1915, xx, 433; xxii, 331.

than the acid (II) yielding the optically active adipic acid. This is seen from the following:



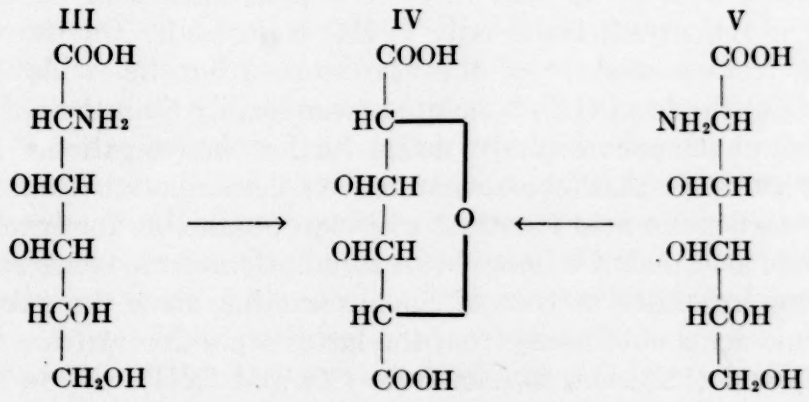
In reality when the brucine salts of the two acids were prepared it was found that the salt of the acid assumed to have the configuration (I) had a lower levorotation than that of (II).

In view of this discrepancy it was decided to reinvestigate the hexosazone obtained from chondrosamine. It was prepared in the same manner as originally, but was recrystallized out of boiling water containing enough pyridine to complete solution. Two recrystallizations sufficed to yield a perfectly pure product which under the microscope consisted of very elongated thin plates with pointed ends and macroscopically had the appearance of bright, glistening scales. This product was suspended in a little alcohol (99.5 per cent) and ether, allowed to stand about 1 hour, and filtered. The dry osazone melted at 201°C. and decomposed at 202°C. Mixed with the osazone obtained from either galactose or from galactosamine, it showed no depression of melting point. Furthermore, pure altrosazone, previously analyzed, which kept its bright lemon yellow color for 1 year without change, was recrystallized out of water and pyridine and then transferred into a mixture of ether and alcohol. It immediately dissolved and on standing, crystallized in long, very fine needles. The melting and decomposition point remained unchanged, 178° and 189°C. respectively. Also the character of mutarotation of osazone of chondrosamine was the same as that of galactosazone. Hence there was no doubt that chondrosamine yielded galactosazone.

We then turned to the two inactive anhydrotetrahydroxya-

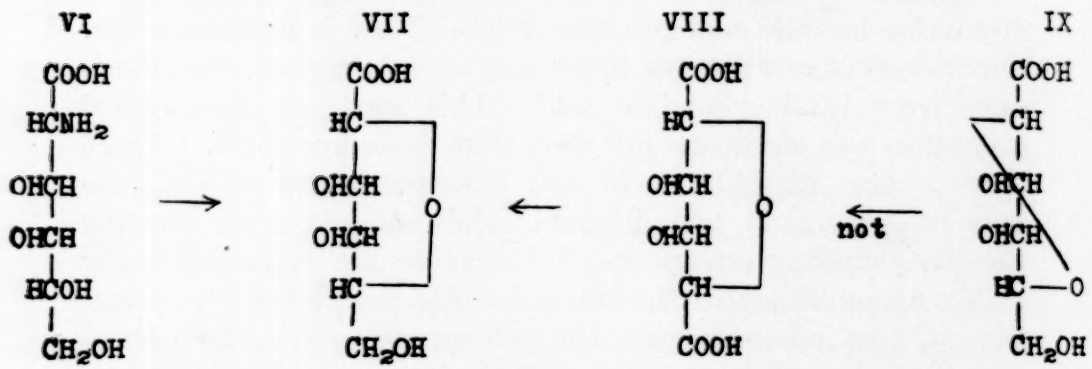
dipic acids. Originally it was found that they differed in their melting points by about 2°. The one obtained from chondrosamine was very slightly colored. It is now recrystallized once more out of acetone. Parallel melting points were made of this substance and a mixture of this and of the anhydromucic acid (obtained from lyxohexosaminic acid) had the identical melting point between 202° and 203°C. (corrected).

It seems on the basis of the present evidence that chondrosamine has the configuration of one of the lyxohexosamines. Fortunately it was possible to prepare a lyxohexosamine synthetically from lyxohexosaminic acid. This sugar in its physical properties was distinctly different from chondrosamine. Hence there is here an instance of two different sugars yielding the same osazone, and two different α-hexosaminic acids yielding the same anhydromucic acid. The facts are explained easily on the assumption that the two pairs of substances are epimeres; that is, that one sugar has the configuration of galactosamine and the other of talosamine, and that the two α-hexosaminic acids have the corresponding configuration. Hence one could assume that in the process of formation of anhydromucic acid one of the hexosaminic acids undergoes a Walden inversion while the other keeps its original configuration. This is seen from the following:



In reality, however, the phenomenon cannot be dismissed by this assumption, and for the following reason. If lyxohexosaminic acid on desamination and subsequent oxidation with nitric acid leads to anhydromucic, then on desamination without

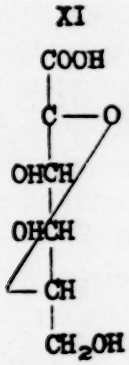
oxidation one should expect the formation of anhydrogalactonic acid. However, when it was attempted to obtain this acid in form of a brucine salt, a substance was obtained which seemed identical with the brucine salt obtained from chondrosamine by desamination and by subsequent oxidation with bromine. This experience seemed puzzling, since it is hard to conceive of a change in configuration brought about by the nitric acid oxidation.



The fact is that starting with the same lyxohexosaminic acid one obtains on one hand anhydromucic acid (VIII), on the other anhydrotalonic (IX) which cannot be converted into (VIII). Of course there is the possibility that on desamination of lyxohexosaminic acid both (VII) and (IX) are formed. On further oxidation with nitric acid there is a possibility that substance (IX) is destroyed, hence only (VIII) is formed. On the other hand, from a mixture of the two monocarboxylic acids (VII) and (IX) the form (IX) is isolated more readily than the epimere.

The phenomenon surely needs further investigation. However, the fact that chondrosamine on desamination leads to a monocarboxylic acid identical with one obtainable for lyxohexosaminic acid makes untenable an assumption of the configuration of chondrosamine as that of ribohexosamine, since the anhydrohexonic acids obtainable from the latter are different from forms (VII) and (IX); this is seen from (X) and (XI):

XI



For the further study of the configuration of chondrosamine, as in the case of glucosamine, it seemed to us necessary to prepare the chondrosaminoheptonic acid. The theoretical reasons were given in the note on glucosaminoheptonic acid. Great difficulties were encountered in crystallizing the chondrosaminoheptonic acid. However, after many failures the chondrosaminoheptonic acid was obtained in the form of large elongated macroscopic prisms with pointed ends.

Finally it was found desirable to simplify the method of preparation of chondrosamine, so as to render the material more accessible and less costly.

EXPERIMENTAL PART.

Preparation of Chondrosamine.—Nasal septums, freed from bone and adhering tissue, were passed through a meat chopper. About 20 pounds of this material were suspended in 30 liters of a hot 2 per cent solution of potassium hydrate. In course of 2 days nearly all the cartilage was dissolved. The solution was then rendered slightly acid by means of acetic acid and neutralized by an excess of barium carbonate. It was concentrated to about one-third of the original volume by immersing into it a steam coil. When sufficiently concentrated, and when most of the protein material was coagulated, the mixture was filtered on suction funnel provided with a layer of kieselguhr. To the solution basic lead acetate was added as long as a precipitate formed. The lead salt was washed by decantation (about ten times), and then filtered over suction. The substance in this

state contained about 75 per cent of water. Portions of the salt of about 2 kilos each were taken up in precipitating jars and a minimum amount of glacial acetic acid was added so as to bring about a solution of the lead salt. The process was facilitated by continuous stirring with a mechanical stirrer. When solution was completed an excess of glacial acetic acid was added until precipitation of the chondroitin sulfuric acid was completed. The precipitate was then filtered, once more treated with glacial acetic acid, again filtered, and then suspended in 95 per cent alcohol. This operation was once repeated. The product was finally filtered and dried on water bath. The process required only 3 liters of glacial acetic acid. 400 gm. of the dry substance were taken up in 1,600 cc. of a 20 per cent solution of hydrochloric acid, 40.0 gm. of stannous chloride and 100.0 gm. of barium chloride added, and all was heated with return condenser over a free flame for 12 hours. When the hydrolysis was completed the reaction product was filtered. The filtrate was concentrated under diminished pressure to a thick syrup. This was taken up in 800 cc. of water and the barium removed by means of sulfuric acid. The final filtrate was concentrated under diminished pressure to a thick syrup. This was taken up in about 100.0 cc. of methyl alcohol, and ether was added very cautiously until the sugar began to crystallize. The crystallization progressed continually for about 24 hours. The yield was about 35–40.0 gm. of chondrosamine chlorhydride.

Chondronic (Anhydrotalonic) Acid. Experiment I.—15.0 gm. chondrosamine hydrochloride were taken up in 75.0 cc. of water, 15 gm. of silver nitrite added, the solution being kept in ice alcohol mixture at the beginning of the reaction, and then allowed to stand over night. The following morning the excess of silver was removed by means of hydrogen sulfide, then 35.0 gm. of bromine were added, and the mixture was allowed to stand 40 hours. The solution was concentrated under diminished pressure to remove bromine, the hydrobromic acid was removed by lead carbonate and silver carbonate, and the excess of silver by means of hydrogen sulfide. The solution was then boiled with brucine. On cooling, the excess of brucine was removed by filtration. The filtrate was concentrated to a syrup and allowed to crystallize. The precipitate was filtered, washed with alcohol, dissolved in

a minimum amount of hot water, and alcohol (99.5 per cent) was added until crystallization began.

In this stage the substance had practically the same rotation as found previously by Levene and La Forge;[2] namely,

$$[\alpha]_D^{20} = \frac{-1.28^\circ \times 2.5876}{1 \times 0.2016 \times 1.03} = -15.94^\circ$$

(the sign + in the earlier paper is a typographical error).

After repeated fractionation from alcohol the rotation was reduced to the following:

$$[\alpha]_D^{20} = \frac{-0.32^\circ \times 2.0329}{1.0 \times 0.0521} = -12.45^\circ$$

The substance had the following composition.

0.0970 gm. substance dried in a vacuum desiccator over sulfuric acid at the temperature of water vapor gave 0.2154 gm. CO_2 and 0.0558 gm. H_2O.

	Calculated for $C_{28}H_{42}N_2O_{19}$:	Found:
C	60.81	60.56
H	6.34	6.43

Experiment II.—It was found advisable to remove the nitric acid in all those experiments where either nitric or nitrous acid were used in the process of preparation of a substance. This procedure facilitated crystallization of the desired substance and improved the yield. The nitric acid was reduced by zinc dust in a sulfuric acid solution, continuing the reaction as long as a test of the solution still showed the presence of nitric acid (brucine test). This operation was introduced as a routine in the preparation of the mono- and dicarboxylic sugar acids.

10.0 gm. of chondrosamine hydrochloride were taken up in 75.0 cc. of water, a few drops of hydrochloric acid added, and 10.0 gm. of silver nitrate were added in small portions. The reaction mixture was agitated by a mechanical stirrer, and the reaction vessel was kept immersed in a cooling mixture. After addition of all the silver the material was allowed to stand over night. The following morning the solution still contained 0.150 gm. of amino nitrogen; hence 2.0 gm. of silver nitrite and 2.0

[2] Levene and La Forge, *J. Biol. Chem.*, 1914, xviii, 130.

cc. of hydrochloric acid (sp. gr. 1.19) were added and the mixture was allowed to stand 2 hours. The filtrate was freed from silver, the volume brought to 100.0 cc., and 25.0 gm. of bromine were added. The bromine was allowed to act for 60 hours with occasional shaking. The solution was then concentrated to half of the original volume and reduced with zinc and sulfuric acid. Finally it was freed from zinc and from mineral acids and converted into the brucine salt of chondronic acid. The excess of brucine was removed by means of chloroform. The aqueous solution was concentrated nearly to dryness. On cooling, it turned into a solid mass. This was recrystallized three times out of 99.5 per cent alcohol. The substance crystallized in form of heavy short prisms (there were among them many typical octahedrons) and melted at 218°C. (corrected), and decomposed at 223°C.

Its optical rotation was as follows:

$$[\alpha]_D^{20} = \frac{-0.34^\circ \times 2.0130}{1.0 \times 0.0550} = -12.4^\circ$$

The substance, dried to constant weight under diminished pressure at the temperature of water vapor, had the following composition.

0.0990 gm. substance gave 0.2210 gm. CO_2 and 0.0590 gm. H_2O.

	Calculated for $C_{29}H_{28}N_2O_{10}$:	Found:
C	60.61	60.68
H	6.34	6.66

Epichondronic (Anhydrogalactonic?) Acid.—10.0 gm. of chondrosaminic acid were taken up in 80.0 cc. of water. 8.0 gm. of silver nitrite and 20.0 cc. of 10 per cent hydrochloric acid were added. The mixture was allowed to stand over night. In course of the next day two portions of 4.0 gm. of silver nitrite and of 10 cc. of 10 per cent hydrochloric acid were added. By the end of the day desamination was complete. The silver was removed by means of hydrogen sulfide, and the filtrate from the sulfide reduced by means of zinc and sulfuric acid. The final solution was boiled ½ hour with excess of brucine. The excess was removed by means of chloroform. The brucine salt was finally

purified by recrystallization out of 99.5 per cent alcohol. The substance crystallized in form of long heavy prisms and melted at 244°C. (corrected).

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.23 \times 2.0304}{1.0 \times 0.0506} = -9.23°$$

The dried substance had the following composition.

0.1004 gm. substance gave 0.2246 gm. CO_2 and 0.0397 gm. H_2O.

	Calculated for $C_{29}H_{28}N_2O_{10}$:	Found:
C	60.61	61.00
H	6.34	6.65

Desaminized Lyxohexosaminic Acid.—10.0 gm. of lyxohexosaminic acid were desaminized following the same procedure as with chondrosaminic acid. The brucine salt did not change its melting point, nor rotation, during the last two recrystallizations. The total number of recrystallizations out of 99.5 per cent alcohol was five. Microscopically the crystals were undistinguishable from those of the brucine salt of chondronic acid. The melting and decomposition points were the same respectively, 218° and 223°C. A mixed melting point of the two substances taken at the same time was identical with that of the individual substances.

The substance had the following rotation:

$$[\alpha]_D^{25} = \frac{-0.30° \times 1.9926}{1.0 \times 0.0500} = -11.96°$$

The substance had the following composition:

0.1040 gm. substance, dried in a vacuum drying apparatus at the temperature of water vapor, gave 0.2219 gm. CO_2 and 0.0575 gm. H_2O.

	Calculated for $C_{29}H_{28}N_2O_{10}$:	Found:
C	60.61	60.27
H	6.34	6.41

Osazone of Chondrosamine.—4.0 gm. of chondrosamine hydrochloride were dissolved in 400.0 cc. of water, and enough sodium acetate was added to neutralize the hydrochloric acid. To this

solution were added 10.0 gm. of phenylhydrazine dissolved in glacial acetic acid. The solution was then immersed for 5 hours into a boiling water bath. A considerable precipitate of osazone appeared during the process of heating. A voluminous precipitate formed on cooling over night. The precipitate was then filtered, washed, and suspended in 500 cc. of hot water. The flask was placed over a flame and pyridine added in small portions until osazone dissolved. The operation was repeated once. Under the microscope the osazone consisted of long plates with pointed ends. No impurity could be noticed on the microscopic slide. The precipitate was filtered on a suction funnel, washed with a very small portion of alcohol, and then transferred into a solution of equal parts of alcohol and ether, allowed to stand for several hours in refrigerator, and filtered. It had the appearance of very light orange glistening plates. It melted at 201° and decomposed at 202°C. A mixed melting point with galactosazone was identical with the original. Also a mixed melting point with the osazone obtained from synthetic lyxohexosamine was identical.

For the optical rotation only 0.050 gm. were used, since it was found that by employing 0.100 gm. in the Neuberg pyridine-alcohol solution one was not certain of accomplishing rapidly a complete solution. A parallel experiment was made with the osazone obtained from the synthetic lyxohexosamine.

The initial rotation in a 0.5 dm. tube was							$\alpha =$	$+0.36°$
After 24 hours	"	"	"	"	"	"	$=$	$0.00°$
" 40	"	"	"	"	"	"	$=$	$-0.10°$
" 80	"	"	"	"	"	"	$=$	$-0.25°$
" 96	"	"	"	"	"	"	$=$	$-0.30°$

Observations were interrupted at this point.

Chondrosaminoheptonic Acid.—38.0 gm. of chondrosamine hydrochloride were taken up in 30.0 cc. of water, 12.0 cc. of 80 per cent prussic acid were added, and the hydrochloric acid was neutralized by means of 17.0 cc. of strong ammonia. The flask was allowed to stand from 5 to 7 days. The reaction product was taken up in 1 liter of water and concentrated under diminished pressure to a thick syrup. The residue was again taken up in water and again concentrated. This operation was repeated four times. Finally it was treated with a solution of

basic lead acetate and with baryta water as long as a precipitate formed. This was washed by decantation ten times, then it was filtered, suspended in water, and decomposed by means of hydrogen sulfide. The filtrate from the sulfide was concentrated to about 400 cc. and then boiled with copper carbonate. The solution of the copper salts, perfectly blue in color, was concentrated to a thick syrup. At this stage the copper salt was precipitated by means of alcohol in pure form, though still amorphous.

0.1034 gm. substance, on combustion on a modified Dennstedt furnace, gave 0.1114 gm. CO_2, 0.0392 gm. H_2O, and 0.0290 gm. CuO.

	Calculated for $C_7H_{13}NO_7Cu$:	Found:
C	29.30	29.38
H	4.57	4.24
Cu	22.17	22.77

However, the substance, although obtained in identical manner, did not always give the same good agreement with the theory.

The copper salts obtained from five experiments were dissolved in water, freed from copper, and the solution was concentrated under diminished pressure to a syrup. This was gradually transferred into 99.5 per cent alcohol which was kept agitated by means of a mechanical stirrer. A white powder formed, which after washing with alcohol and ether and drying was no longer hygroscopic. The precipitate was suspended in 750 cc. of alcohol (99.5 per cent) and hydrochloric acid gas was passed in, in a lively stream. Part of the acid dissolved. The greatest part, however, remained insoluble. After some time a flocculent precipitate of the ester appeared, which again disappeared, and a second permanent precipitate of the lactone settled out.

The reaction product was allowed to stand over night. The following day the supernatant liquid and the flocculent precipitate of the lactone could be easily removed. The remaining alcohol-insoluble part was dissolved in water and the hydrochloric acid was removed by means of lead carbonate and silver carbonate. The final solution was concentrated to about 100.0 cc. and methyl alcohol was added in very small portions until there appeared a permanent opalescence. On standing over night an oil settled out. The supernatant liquid was decanted, and

each fraction allowed to stand separately. After 3 days' standing in the supernatant liquid fraction there appeared a rosette composed of prisms with needle-shaped points. The crystals were about 3 mm. long. From that moment crystallization proceeded rapidly. The yield was 5.0 gm. Soon also the oil began to crystallize and in course of a day solidified to a crystalline mass. It was filtered, dissolved in little water, and methyl alcohol was added in small portions until the acid began to crystallize. The yield was 4.0 gm. The original crystal form did not change on recrystallization. The air dry acid lost approximately one molecule of water on drying in a vacuum drying apparatus at the temperature of chloroform vapor. When heated at a higher temperature it continued losing weight indefinitely, at the same time changing color. The substance melted at 136° C. (corrected) and had the following composition.

0.1006 gm. air dried substance gave 0.1292 gm. CO_2 and 0.0639 gm. H_2O.

0.1500 gm. substance employed for Kjeldahl nitrogen estimation required for neutralization 6.00 cc. 0.1 N acid.

	Calculated for $C_7H_{15}NO_7+H_2O$:	Found:
C	34.59	34.82
H	7.00	7.11
N	5.76	5.60

The substance had the following optical rotation in 2.5 per cent hydrochloric acid.

Initial.

$$[\alpha]_D^{20} = \frac{-0.29° \times 2.1265}{1.0 \times 0.0516} = -11.95°$$

Equilibrium.

$$[\alpha]_D^{20} = \frac{-0.35° \times 2.1265}{1.0 \times 0.0516} = -14.40°$$

THE SYNTHESIS OF HEXOSAMINES. I.

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The need of the synthesis of hexosamines became very evident after the discovery of the new sugar chondrosamine. The explanation of its structure was facilitated by the synthesis of one lyxohexosamine, but the theory of its structure will be proven beyond dispute after either a ribohexosamine or the other lyxohexosamine are synthesized. Unfortunately it will be a very difficult task to complete the series of theoretically possible aminohexoses. After addition of prussic acid to the pentosimines there appears only one form of hexosaminic acid, or the equilibrium of the two is so much in favor of one that the isolation of the other is not possible.

There is recorded only one successful attempt to prepare synthetically a hexosamine. Fischer and Leuchs[1] prepared glucosamine. They identified the sugar by its osazone and by the phenylisocyanate derivative. Neither the free sugar nor a simple salt of it have been obtained, because of the difficulty of the task.

The difficulty lay in the fact that the reaction product containing the sugar contained also a great excess of sodium sulfate and the unreduced lactone, and perhaps some of the unchanged amino-acid. The presence of the amino-acid and of its lactone hinders the crystallization of the acyl derivatives, while the presence of sodium sulfate renders the isolation of a salt of the amino sugar very difficult at the best.

It was found more convenient to carry out the reduction of the lactones in a hydrochloric acid solution. Both sodium and potassium amalgam were used as reducing agents. This mode of procedure proved very effective at least for two sugars, gluco-

[1] Fischer, E., and Leuchs, H., *Ber. chem. Ges.*, 1903, xxxvi, 24.

samine and lyxohexosamine. It did not improve conditions in the isolation of xylohexosamine.

The first experiments in the reduction of glucosaminic acids were accomplished by means of electrolytically prepared potassium amalgam. Indeed, in the very first experiment it was possible to isolate a small quantity of glucosamine containing only 2.5 per cent of ash. However, the same conditions could not be duplicated for the present, and all the later reductions and all the reductions of the lactones of lyxohexosaminic and of xylohexosaminic lactones were accomplished by means of a 2.5 per cent sodium amalgam.

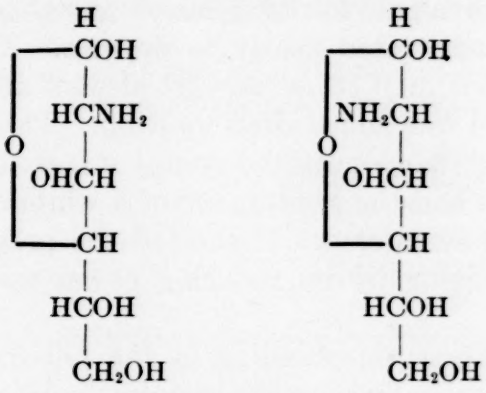
Glucosamine.—The reduction of glucosaminic acid was carried out principally for the sake of method. As already stated, when potassium amalgam was employed as reducing agent and methyl alcohol used for fractionation, a fraction was obtained which contained only 2.5 per cent potassium chloride. When sodium amalgam was employed it was possible to separate the reaction product into one fraction which contained besides the sugar only sodium chloride, leaving all the unchanged lactone, amino-acid, and part of the sugar in solution. The first fraction contains approximately 50 per cent of the sugar present in the reaction mixture.

From the first fraction the isolation of a pure benzoyl derivative offers no difficulty at all, and the pentabenzoyl derivative of glucosamine was obtained in good yield. On the contrary, from the second fraction containing 50 per cent of the sugar only a fraction of a gram of the benzoyl derivative was isolated. The benzoyl derivative of course is readily converted into the free sugar.

Xylohexosamine.—The lactone was reduced in hydrochloric acid solution with sodium amalgam. For the present no successful fractionation of the reaction product was accomplished. The hydrochloride of the sugar is apparently very soluble in methyl alcohol. The sugar was identified by the osazone which possessed the melting point and the optical rotation of gulosazone. The pentabenzoyl derivative was obtained in rather poor yield. The yield of the naphthylisocyanate derivative was also rather poor; hence its purification was not accomplished for the present.

Lyxohexosamine.—The synthesis of this sugar was of immediate

importance in view of its bearing on chondrosamine. By recrystallizing the osazone of chondrosamine from pyridine and water it acquired the properties of galactosazone. Also the lyxohexosamine gave an osazone undistinguishable from galactosazone. Furthermore, the anhydromucic acid obtained from lyxohexosamine and from chondrosamine seem to be identical. On the other hand, the hydrochlorides of the two amino sugars were totally different in their melting points and their optical rotation. On the basis of the existing evidence the two sugars appear as epimeres of the following structure.



EXPERIMENTAL PART.

Glucosamine.—For the preparation of all lactones of hexosaminic acids it is important to start with very pure material. The glucosaminic acid used in these experiments was recrystallized several times out of water. 5.0 gm. lots of the acid were taken up in 50.0 cc. of 99.5 per cent alcohol. Dry hydrochloric acid gas was passed in, in a lively stream. The acid soon dissolved. After a while a flocculent precipitate begins to appear; if the solution is actively shaken during this phase nearly all the flocculent precipitate of the esters disappears. For a while the solution remained clear and then turned quite opaque. At this stage it was found advisable to immerse the flask in a cooling mixture and to continue passing the gas. Soon a precipitate of the lactone appeared. This was allowed to stand over night in the refrigerator. One can wash the lactone by decantation with 99.5 per cent alcohol and with dry ether and finally filter the lactone and dry it in a vacuum desiccator. It is then possible to

continue the work on the pure lactone. However, it was found that this mode of procedure had no advantage over the less troublesome method of Fischer and Leuchs. In most of the experiments the latter method was followed.

Isolation of Glucosamine Hydrochloride.—5.0 gm. of the lactone hydrochloride were reduced with 40.0 gm. of potassium amalgam (2 per cent), hydrochloric acid being used for neutralization. The reduction was carried out in a vessel immersed in a cooling mixture and provided with a mechanical stirrer. At the end of the experiment the solution tested with Fehling's reagent showed a reduction equivalent to 1.9 gm. of glucose. The reaction product was concentrated nearly to dryness. The residue was taken up in about 30.0 cc. of methyl alcohol and concentrated hydrochloric acid was added drop by drop. The flask was carefully warmed over flame until the syrupy material was all in solution and the salt had the appearance of a white crystalline powder. The salt was filtered. It showed the presence of considerable sugar, judging by its reducing power towards Fehling's solution.

The mother liquor on standing in the refrigerator formed a precipitate of practically pure glucosamine hydrochloride.

0.1030 gm. substance gave 0.1246 gm. CO_2, 0.0600 gm. H_2O and 0.0026 gm. (2.55 per cent) ash.

	Calculated for $C_6H_{13}NO_5.HCl$:	Found:
C	33.40	33.85
H	6.54	6.69

In a few other experiments the sugar contained more salt. This experiment goes to show that there can be found favorable conditions for the isolation of glucosamine hydrochloride directly from the reaction product.

Preparation of the Pentabenzoyl Derivative.—The material used for this preparation was obtained from several reduction experiments. Potassium and sodium amalgam were used for reduction and hydrochloric acid for neutralization. The reaction product was treated as in the above experiment. The fraction containing only sugar and salt was used for benzoylation.

6.5 gm. of the sugar (estimated by means of Fehling's solution) were taken up in 140.0 cc. of water, 25.0 cc. of benzoyl chloride,

and 40.0 cc. of a 50 per cent sodium hydrate solution. It was shaken for about 1½ hours with constant cooling, and then placed in a shaking machine over night. The benzoyl derivative, which had the appearance of dry white balls, was washed with water, then dissolved in chloroform, and shaken with water in a separatory funnel. Finally the chloroform solution was dried over sodium sulfate, filtered, and concentrated to dryness. The residue was recrystallized out of 4 liters of 98 per cent alcohol. On cooling, the substance crystallized in long colorless needles. The mother liquor on concentration gave a second precipitate. The substance melted at 216°C. and had the following composition.

0.1012 gm. substance gave 0.2606 gm. CO_2 and 0.0453 gm. H_2O.
0.2530 " " required for neutralization by the Kjeldahl method 5.05 cc. 0.1 N acid.

	Calculated for $C_6H_8NO_5(C_6H_5CO)_2$:	Found:
C	70.38	70.23
H	4.73	5.00
N	2.00	1.99

The optical rotation of the substance in pyridine was as follows:

$$[\alpha]_D^{20} = \frac{+0.58^\circ \times 1.9992}{0.5 \times 0.0522} = +44.4^\circ$$

The benzoyl derivative has been converted into the hydrochloride of glucosamine by previous workers; hence it was not considered important to repeat it on this occasion.

Xylohexosamine.—The lactone was prepared in the same manner as that of glucosaminic acid. Also in the reduction the general plan was followed. However, all the attempts to obtain a fraction free from the lactone were for the present unsuccessful. When hydrochloric acid was used for neutralization the salt always separated out free from sugar, and when sulfuric acid was employed for the same purpose salt, lactone, and sugar crystallized in the same fraction.

A solution containing about 6.0 gm. of the amino sugar and 1.0 gm. of amino nitrogen was taken up in 500 cc. of water to which 80.0 cc. of benzoyl chloride and 110 cc. of a 50 per cent sodium hydrate solution were added. The benzoylation proceeded as in the glucosamine experiment. The final product was taken up

in methyl alcohol and ether was added as long as it caused the precipitation of an oil. The supernatant liquid was decanted and allowed to evaporate spontaneously. A precipitate of white needles formed. It was filtered off and recrystallized once out of 99.5 per cent alcohol. It melted at 162°C. and had the following composition.

0.1012 gm. substance gave 0.2632 gm. CO_2 and 0.0416 gm. H_2O.

	Calculated for $C_6H_8NO_5$ $(C_6H_5CO)_3$:	Found:
C	70.38	70.93
H	4.73	4.60

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{+1.1^\circ \times 1.9422}{0.5 \times 0.0550} = +77.6^\circ$$

Osazone of Xylohexosamine.—A solution containing 2.5 gm. of the amino sugar was taken up in 250 cc. of water and 6.0 gm. of phenylhydrazine dissolved in glacial acetic acid were added. The flask containing the solution was then placed in boiling water bath for 4 hours. On cooling, the osazone settled out. It was recrystallized four times out of water and pyridine. It then consisted uniformly of curved needles. The substance was taken up in a very little alcohol and ether. It dissolved and crystallized on evaporation of the solvent in the form of bright lemon yellow precipitate. It melted at 173° (corrected) and decomposed at 185°C.

The substance had the following composition.

0.1000 gm. substance gave 0.2208 gm. CO_2 and 0.0560 gm. H_2O.

	Calculated for $C_{18}H_{24}N_4O_4$:	Found:
C	60.33	60.21
H	6.14	6.22

The rotation of the substance was as follows:

0.1000 gm. substance dissolved in 5.0 cc. of Neuberg's alcohol-pryidine solution had an initial rotation of $\alpha = +0.07^\circ$ and after 40 hours $\alpha = +0.45^\circ$.

The napthylisocyanate derivative was not obtained in pure form. We intend to report on the phenyl and napthylisocyanate derivatives of the hexosamines on another occasion.

Lyxohexosamine.—15.0 gm. of lxyohexosaminic acid were taken up in three small flasks, each containing 5.0 gm. of the acid and 50.0 cc. of 99.5 per cent alcohol. Into each a lively stream of dry hydrochloric acid gas was passed. The formation of the lactone proceeded in the same way as with the glucosaminic acid. The flasks were allowed to stand over night, then transferred to a distilling flask by the aid of distilled water. 150.0 gm. of 2.5 per cent sodium amalgam were used for reduction. At the end of the experiment the solution tested with Fehling's reagent showed a reduction equivalent to 6.5 gm. of glucose.

The product of reaction was concentrated under diminished pressure to dryness. The residue was taken up in 75.0 cc. of methyl alcohol and concentrated hydrochloric acid added drop by drop until all the syrup dissolved and sodium chloride appeared in the form of white crystalline powder. This first precipitate showed no reducing power at all.

The filtrate was again concentrated to dryness and taken up in 99.5 per cent ethyl alcohol and hydrochloric acid as before. On standing, an oily mass separated out. In course of the night this turned to a crystalline mass. The supernatant liquid was decanted and the crystalline mass triturated with methyl alcohol. It consisted of long heavy prisms with needle-shaped points. The substance burned as carbohydrates do and left behind a small amount of ash. The liquid which had been decanted off the crystalline mass on standing began to form on the walls and on the bottom of the flask a crystalline deposit consisting of long prisms of the same appearance as those of the crystalline mass. On burning it left practically no ash.

The substance was redissolved in a little water and 99.5 per cent alcohol added in small portions until on scratching the sugar began to crystallize. The crystals had the same appearance as on first crystallization. The substance melted at 185°C. (corrected), and had the following rotation:

Initial.

$$[\alpha]_D^{25} = \frac{+1.5^\circ \times 2.0269}{1.0 \times 0.0485} = +62.69^\circ$$

Equilibrium.

$$[\alpha]_D^{25} = \frac{+2.18^\circ \times 2.0269}{1.0 \times 0.0485} = +91.10^\circ$$

0.1050 gm. substance dried in a vacuum desiccator over sulfuric acid at 25°C. gave 0.1284 gm. CO_2 and 0.0604 gm. H_2O.

0.0244 gm. substance gave 2.94 cc. N at 22°, 762 mm.

0.0820 " " required 3.7 cc. 0.1 N $AgNO_3$ solution.

	Calculated for $C_{12}H_{13}O_5N.HCl$:	Found:
C	33.40	33.35
H	6.54	6.39
N	6.51	6.74
Cl	16.45	15.98

The mother liquor of the crystalline mass was concentrated under diminished pressure. It contained 3 gm. of sugar and was taken up in 400 cc. of water, 8.0 gm. of phenylhydrazine dissolved in glacial acetic acid were added, and the flask containing the solution was immersed in a boiling water bath for 4 hours. The osazone began forming while the solution was still heated. On cooling, a voluminous precipitate of osazone deposited. The osazone was taken up in 400.0 cc. of boiling water and pyridine was added gradually (the water was kept boiling) until the solution was complete. After three recrystallizations microscopic slides showed perfectly formed elongated plates with pointed ends, and an absolute absence of oily droplets. The osazone was then filtered on a suction funnel, transferred into a mixture of equal parts of alcohol and ether, and filtered. It then had the appearance of light orange glistening plates.

It melted at 201°C. and decomposed at 202° (corrected). Mixed with osazone from chondrosamine and with galactosazone it showed the same melting and decomposition points.

0.1006 gm. substance gave 14.2 cc. N at 29° and 757 mm.

	Calculated for $C_{18}H_{24}N_4O_4$:	Found:
N	15.64	15.80

For the rotation of the substance 0.050 gm. was dissolved in 5.0 cc. of Neuberg's pyridine and alcohol mixture. The initial rotation was +0.36°, after 24 hours, +0.03°. (It was not followed further.)

Another sample of osazone which had been recrystallized out of 85 per cent alcohol and which dissolved more readily, though with considerable difficulty, showed the following rotation:

0.100 gm. substance dissolved in 5.0 cc. of alcohol-pryidine solution gave an initial rotation of +0.56°, and an equilibrium of +0.16°.

From a smaller experiment a benzoyl derivative was obtained in the same manner as from xylohexosamine. However, the quantity was too small for recrystallization.

THE PHYSIOLOGICAL ACTION OF GLUCAL.

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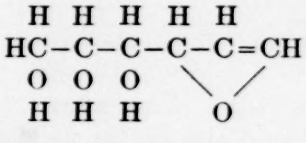
The exact nature of the intermediate forms through which glucose passes before it is finally broken up by the animal body during metabolism is still a matter of question and needs further investigation. In view of this fact it was thought highly essential that a substance like glucal, $C_6H_{10}O_4$, which is so closely related to glucose, in fact derived from it, be studied in reference to its behavior in the animal body. The possibility that glucal may be an intermediate stage of glucose metabolism is supported by the facts that glucal is derived from glucose by reduction and dehydration and that it is quite unstable. Nef[1] states that the structural formula of glucal is:



If this formula is correct it lends support to the theory that glucal may be an intermediate product of glucose metabolism, for a substance possessing such a structure by its very nature indicates that it may be readily transformed into other derivatives.

It may also be mentioned here that Feulgen[2] suggests glucal as the carbohydrate of nucleic acid.

The work and the results recorded here by no means settle the question whether or not glucal is an intermediate product of glucose metabolism. However, a number of positive results have been obtained which may prove of assistance in future work on this problem.

[1] Nef, J. U., *Ann. Chem.*, 1914, cdiii, 334.

[2] Feulgen, R., *Z. physiol. Chem.*, 1914, xcii, 154.

The Preparation of Glucal.

The glucal used in the experiments recorded was prepared according to the methods given by Fischer[3] except for the saponification of the acetyl groups of the acetyl derivative.

According to Fischer the saponification of the acetyl groups of the triacetyl glucal is carried out with barium hydroxide. The barium is removed by precipitation with sulfuric acid and filtering. Glucal is very unstable in an acid solution, so that great precaution must be taken not to make the solution acid with an excess of sulfuric acid. In taking this precaution lies the danger that not all the barium is removed and so may be present in the final product. Barium itself is toxic.

In order to obviate the possibility of barium poisoning, then, the triacetyl glucal was saponified with sodium hydroxide. The exact procedure was this: 5 gm. of dry crystalline triacetyl glucal were finely powdered and treated with 130 cc. of 0.5 N sodium hydroxide. This mixture was shaken on a machine for 2 hours. To the solution were then added 130 cc. of 0.5 N sulfuric acid. Thus far the process was carried out at room temperature. The solution then was distilled under reduced pressure at 40°C. till the separation of salts occurred. The glucal then was extracted three times with 100 cc. portions of absolute alcohol. The latter was distilled off under reduced pressure, leaving the final product, glucal, as a colorless syrup. This again was taken up with 100 cc. of absolute alcohol and the latter distilled off as before. The syrup was then dissolved in water which had been boiled for 10 minutes. Thus a sterile solution of glucal was prepared. Any acidity due to incomplete removal of acetic acid was neutralized with 0.1 N NaOH. (The trace of sodium acetate thus formed is physiologically inactive.) By this method 5 gm. of triacetyl glucal yielded on an average 2.5 cm. of glucal.

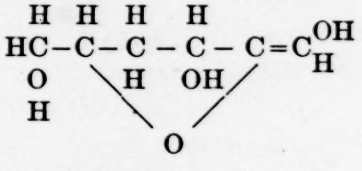
[3] Fischer, E., *Ber. chem. Ges.*, 1914, xlvii, 196.

The Chemical Characteristics of Glucal, Its Molecular Formula, and Its Probable Structural Formula.

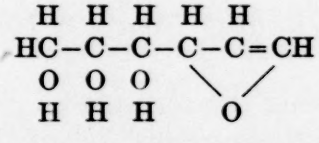
The glucal prepared in the manner described possessed the following characteristics:

1. On treatment with strong mineral acids it formed a dark char.
2. On treatment with strong alkalis it turned yellow.
3. In a water solution a dark green precipitate was formed on the addition of concentrated sulfuric or hydrochloric acid.
4. It could readily be detected in urine by the formation of a dark green precipitate when 1 cc. of the glucal containing urine was diluted with 5 cc. of water, and 2 cc. of concentrated sulfuric acid were added. Samples of urine containing glucal in the proportion of 1 gm. of glucal to 1 liter of urine gave the test well.
5. The glucal recolorized fuchsin sulfurous acid.
6. Iodine and bromine were rapidly absorbed by glucal. The glucal iodine combination did not color a 1 per cent starch solution.
7. The glucal reduced Fehling's and Haines' solutions.
8. On treatment with sodium acetate and acetic anhydride, glucal was transformed into triacetyl glucal.

The molecular formula of glucal is $C_6H_{10}O_4$. There are various opinions as to its structural formula. Fischer suggests that it is constructed in this manner:

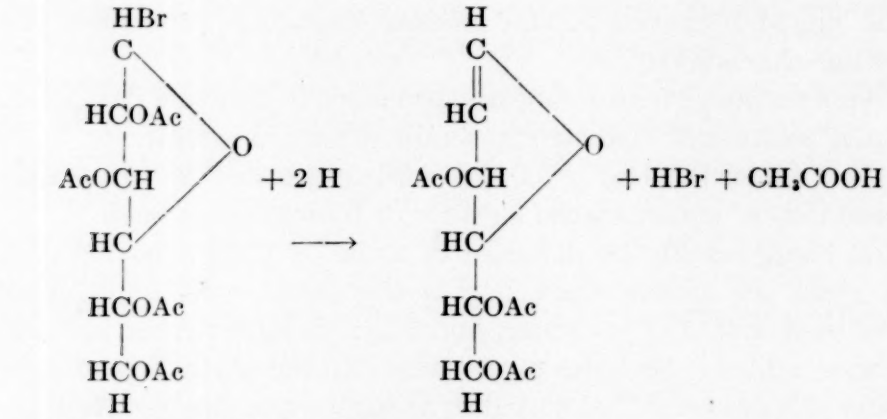


Nef states that it has this structure:



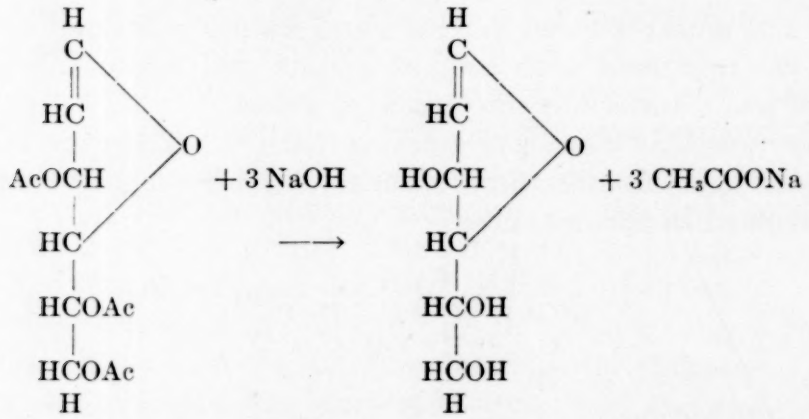
Judging from the character of the reactions which take place in the formation of glucal *in vitro* it would seem that the Nef formula was the correct one. For when acetobromoglucose is treated with zinc, it yields one molecule of hydrobromic acid and one

molecule of acetic acid. We may then represent the reaction thus (Ac = CH_3CO):



Acetobromoglucose. Triacetyl glucal.

The saponification of the acetyl groups of the triacetyl derivative may be represented in this manner:



It may then be assumed that the lactone ring is of an oscillating type because of the instability of glucal. Accordingly the product may be transformed from a γ lactone compound to a β lactone.

Thus a product having the formula as given by Nef would be produced. This formula accounts for all the reactions of glucal as given according to Fischer except the pine splinter reaction indicating a furane ring. However, the chemical composition of the substance in a pine splinter giving the reaction is not definitely known. Consequently, in the opinions of some, a furane

compound may not be the only compound producing green coloration of a pine splinter moistened with hydrochloric acid.

Experiments on the Physiological Action of Glucal.

The first two experiments were conducted solely for the purpose of determining whether glucal is a toxic compound or not. In the first experiment a 2 per cent solution was injected subcutaneously into six frogs as indicated:

Weight.	Dose.	Time.	Results.	
			Immediate.	Later.
gm.	*cc.*			
56	4	2.56	No change.	No change.
42	3	3.00	" "	" "
37	2.5	3.10	" "	" "
Controls, using water instead of glucal solution.				
30	2.6	3.17	No change.	No change.
38	4	3.18	" "	" "
60	5	3.20	" "	" "

The frogs showed no symptoms of poisoning such as morbidity, muscular twitching, or spasms as a result of the injection of glucal. Their behavior at all times was normal.

From these results we may conclude that glucal injected subcutaneously into frogs in doses of 1.4 mg. per 1 gm. of body weight produces no toxic effects.

In the second experiment, a guinea pig weighing 250 gm. was injected subcutaneously with 5 cc. of a 10 per cent solution of glucal. The animal showed no effects of poisoning locally or systemically, immediately or at any time afterwards.

Next an experiment was carried out to determine the effect of glucal on respiration and blood pressure. A dog weighing 8 kilos was anesthetized with ether. A cannula was inserted into the trachea and connected with a respiration tracing apparatus. Also a cannula inserted into the right carotid artery was connected with a blood pressure mercury manometer. Tracings of both movements were taken; *i.e.*, respiration and blood pressure. The

blood pressure was 130 mm. Hg and the respiration normal in each of the following five intravenous injections.

	Time.
5 cc. M/8 Na acetate	3.14
5 " 10 per cent glucal	3.16
5 " 10 " " "	3.21
5 " 10 " " "	3.23

The dog was observed for an hour but no deviation from the normal state occurred.

Also an experiment on the metabolism of glucal in the animal body was carried out in the following manner:

A dog, not anesthetized, weighing 10 kilos, was injected with 6.5 gm. of glucal, with a Woodyatt continuous injection machine, by way of the left saphenous vein at the rate of 0.9 gm. per kilo of body weight per hour. The dog showed no ill effects during the operation or afterwards. The urine, collected by catheter, was examined as indicated:

Test.*	Before glucal injection.	After glucal injection.
H_2SO_4	Pink, clear.	Green precipitate.
Reduction (Haines' solution)	None.	Slight.
H_2SO_4	1 cc. of urine + 0.01 cc. of a 10 per cent solution of glucal diluted with 5 cc. of water gave a green precipitate.	

* Glucose does not give this test with sulfuric acid.

Then we may conclude that glucal injected into the venous system of an animal is not totally metabolized if injected at the rate of 0.9 gm. per kilo per hour. This is the limit of tolerance of the injection of glucose under similar conditions. Whether any of the glucal was metabolized could not be estimated, since thus far no accurate method of determining glucal in solution has been developed. It is hoped that this may be accomplished by bromine or iodine titration.

It is possible that if glucal is formed from glucose in the animal body, a reversible process may also take place in case of an excess over the normal tolerance of glucal, such as has been observed

in the above experiment. Therefore it seemed important that for future work some test for glucose be established distinguishing it from glucal, and not clouded by the reaction of the latter. Since one of the common methods employed for determining the presence of glucose is the fermentation test, it has been of interest to determine whether or not glucal is fermentable.

Accordingly a 1 per cent peptone and 0.3 per cent bouillon broth was prepared. Its reaction was adjusted so that 5 cc. of the broth exactly neutralized 1 cc. of 0.05 N NaOH. 13.5 cc. were placed in each of eighteen fermentation tubes. The tubes were divided into sets of six. To one of the three sets thus formed were added 1.5 cc. of a 10 per cent glucal solution, per tube; to another, 1.5 cc. of a 10 per cent glucose solution, per tube; and to the third, 1.5 cc. of distilled water per tube. (The solutions containing the glucose and those containing the water additions were sterilized together with their respective additions. The tubes later containing glucal were sterilized before the latter was added because of the instability of glucal at high temperatures. The glucal additions, however, were also sterile, since the glucal had been obtained from an alcohol extract and dissolved in water which had previously been boiled for 10 minutes.)

Then three tubes of each set were inoculated, each with one loopful of a pure culture of *Bacillus coli*. The other three tubes of each set were inoculated with one loopful of a pure culture of *Saccharomyces cervisiæ*. The following is a record of the procedure and results (temperature 35°C.):

Tube.	Substance tested.	Organism.	Results. Gas.		
			12 hrs.	24 hrs.	36 hrs.
	cc.				
1	1.5 10 per cent glucal.	*Bacillus coli.*	0	0	0
2	1.5 10 " " "	" "	0	0	0
3	1.5 10 " " "	" "	0	0	0
4	1.5 10 " " "	Yeast.	0	0	0
5	1.5 10 " " "	"	0	0	0
6	1.5 10 " " "	"	0	0	0
7	1.5 10 " " glucose.	*Bacillus coli.*	2 cm.	2.5 cm.	2.5 cm.
8	1.5 10 " " "	" "	2 "	2.5 "	2.5 "
9	1.5 10 " " "	" "	2 "	3 "	3 "
10	1.5 10 " " "	Yeast.	0	3 "	3 "
11	1.5 10 " " "	"	0	2.5 "	2.5 "
12	1.5 10 " " "	"	Bubble.	2.5 "	2.5 "
13	1.5 water.	*Bacillus coli.*	0	0	0
14	1.5 "	" "	0	0	0
15	1.5 "	" "	0	0	0
16	1.5 "	Yeast.	0	0	0
17	1.5 "	"	0	0	0
18	1.5 "	"	0	0	0

There were definite growths of the organisms in all the tubes in 24 hours. The yeast cells of the tubes containing glucal were examined microscopically after 24 hours, and budding forms of the cells could be seen.

The results indicate that glucal is fermented neither by *Bacillus coli* nor by *Saccharomyces cervisiæ*. The fermentation test then may be used in distinguishing between glucose and glucal.

In summary, the above recorded results indicate that:

1. Glucal is not a toxic substance.

2. Glucal produces no effect on blood pressure or on respiration when injected intravenously in moderate quantities.

3. Glucal may be detected in urine with concentrated sulfuric acid.

4. Glucal combines with iodine and the combination does not color a 1 per cent starch solution.

5. The animal body does not completely metabolize glucal when the latter is injected at the rate of 0.9 gm. per kilo per hour.

6. Glucal is not fermented either by *Bacilli coli* or *Saccharomyces cervisæ*.

I wish to express my gratitude for the valuable suggestions and assistance of Dr. Mathews, who suggested the problem, and to Drs. Koch, Wilder, and Woodyatt, and Messrs. Hanke and Theobald.

THE FORMATION OF *d*-LACTIC ACID BY THE AUTOLYSIS OF PUS.

By HIIZU ITO.

(From the Institute of Medical Chemistry of the Kyoto Imperial University, Kyoto, Japan.)

(Received for publication, June 19, 1916.)

That a lactic acid is present in pus was accidentally found by Tatsukichi Irisawa.[1] Though he stated, "Ferner habe ich gelegentlich aus etwa einem Liter frischen Empyemeiters eine kleine Quantitaet Milchsaeure dargestellt," he gave no further description of his work. It was, therefore, impossible to decide what form of the lactic acid was found by him. An attempt was made by the writer to determine the form and to ascertain whether the *d*-lactic acid was formed by the autolysis of pus. The pus obtained from the pleura was used in the experiments.

The method of Professor Yoshikawa was adopted with modifications. An equal quantity of pus was taken in each of two flasks and that in one flask was mixed with five times as much 90–95 per cent alcohol. After standing for 24 hours it was filtered. The residue was thoroughly washed with alcohol. After the alcohol was evaporated, the residue, acidified with phosphoric acid, was extracted as much as possible with ether in Sudo's apparatus. After the ether was evaporated on a water bath, the syrup which remained was boiled with a little water and lead carbonate, filtered, and washed with water. The lead was removed from the filtrate, passing hydrogen sulfide through it. After the filtrate was strongly concentrated on the water bath, lactic acid was extracted with ether. After the ether was evaporated, the lactic acid was transformed into lithium lactate by boiling with water and lithium carbonate. The lactate solution was transferred into a 25 cc. flask and water was added to it to the 25 cc. mark. The solution was shaken and filtered, and examined with the polariscope.[2] The pus of the second flask was digested with toluene and chloroform at 37–38°C. for 4 days. The procedure of extracting the

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[1] Irisawa, T., *Z. physiol. Chem.*, 1893, xvii, 349.

[2] Yoshikawa, J., *Z. physiol. Chem.*, 1913, lxxxvii, 389.

lactic acid from the digested material was the same as with the first portion. But in the last two cases of the series which follow, the method of Mondshein, which was modified by Yoshikawa,[3] was adopted as the method of extracting the lactic acid, because it was suggested to the writer that Mondshein's method would give a better result than the alcohol extraction. In these cases, one portion of the pus was mixed with five times as much water. To precipitate the protein content, the mixture, to which was added a weak acetic acid, was boiled for 30 minutes and filtered through "nutsch." The residue was warmed with 50 cc. of water and 15 cc. of 15 per cent NaOH, which dissolved the former. The solution was then neutralized with weak sulfuric acid and filtered. This filtrate was put together with the other which was prepared through the "nutsch." The whole filtrate was concentrated to a syrup, and was extracted with 95 per cent alcohol, which was entirely evaporated. The residue was strongly acidified with phosphoric acid and was extracted with ether in the extracting apparatus. The lactic acid which was obtained after the ether was evaporated was transformed into lithium lactate by adding water and lithium carbonate. The lithium lactate was treated as suggested by Yoshikawa and was examined with the polariscope. The calculation of the *d*-lactic acid from α was followed exactly as Yoshikawa suggests.

Patient.	Pus in each determination.	Lithium lactate obtained.		Lactate formed by autolysis.
		At once.	After 4 days' autolysis.	
	gm.	*gm.*	*gm.*	*gm.*
I. School boy, 19 years. Pus from chest after rib resection.	300	0.7370	0.8366	0.0996
II. Man, 43 years. Pus from chest after rib resection.	500	0.6388	0.7370	0.0982
III. Boy, 3 years, 1 month. Pus from chest after thoracocentesis.	400	1.1244	1.2295	0.1051
IV. Man, 28 years. Pus from chest after thoracoplasty.	280	0.1906	0.2748	0.0842
V. Man, 63 years. Pus from chest after thoracocentesis.	300	0.2494	0.4373	0.1879
VI. Man, 20 years. Pus from chest after rib resection.	250	0.1653	0.2665	0.1012

[3] Yoshikawa, *Z. physiol. Chem.*, 1913, lxxxvii, 406.

To determine finally that the lactic acid found in both portions was identical with *d*-lactic acid, the lithium lactate which was formed from the material experimented on was transformed into zinc lactate.

Transformation of Lithium Lactate into Zinc Lactate.

The lithium lactate solution which was obtained in the series of the first portion from all six cases was put all together. The solution was strongly acidified with phosphoric acid and was extracted with ether in Sudo's apparatus. After the ether was evaporated, the residue was diluted with water, boiled with pure lead carbonate, filtered, and washed well. The lead was taken away from the filtrate with hydrogen sulfide; the solution with no lead was strongly concentrated on the water bath, and was extracted as much as possible with ether. After the ether was evaporated the residue was boiled with water and pure zinc oxide, filtered, and washed thoroughly. The filtrate with the water used for washing was evaporated on the water bath until the zinc lactate began to crystallize. It was repeatedly recrystallized from hot water, until pure crystals were obtained. The crystals showed under examination the typical characteristics of the *d*-zinc lactate.

The quantity of unhydrated zinc lactate in the solution of 100 cc. was 2.6546 gm.; length of the tube, 2 dm.; rotation, using sodium light, at 19° = −0.438°. Therefore: $[\alpha]_D^{19} = -8.25$.

0.2126 gm.	substance	lost at 110°C.	0.0278 gm.	H_2O.
0.1154 "	"	gave	0.0384 "	ZnO.
0.1322 "	"	lost at 110°C.	0.0168 "	H_2O.
0.1848 "	"	gave	0.0613 "	ZnO.

	Calculated for $(C_3H_5O_3)_2Zn + 2H_2O$:	Found: 1.	2.
H_2O	12.9	13.08	12.71
Zn	26.86	26.69	26.62

The zinc lactate experimented on was thus shown to be identical with *d*-zinc lactate.

The quantity of unhydrated zinc lactate in the solution of 100 cc. was 2.7823 gm.; length of the tube, 2 dm.; rotation, using sodium light, at 15° = −0.446°. Therefore: $[\alpha]_D^{15} = -8.01$.

0.1275 gm. substance lost at 110°C. 0.0165 gm. H_2O.
0.3742 " " gave 0.1246 gm. ZnO.

	Calculated for $(C_3H_5O_3)_2$ Zn + 2 H_2O:	Found:
H_2O	12.9	12.94
Zn	26.86	26.75

The zinc lactate obtained in the second portions was thus shown to be identical with *d*-zinc lactate.

SUMMARY.

1. *D*-lactic acid is a constant constituent of pus.
2. *D*-lactic acid is distinctly increased by the autolysis of pus.

THE ORIGIN OF THE HUMIN FORMED BY THE ACID HYDROLYSIS OF PROTEINS.

II. HYDROLYSIS IN THE PRESENCE OF CARBOHYDRATES AND OF ALDEHYDES.

By ROSS AIKEN GORTNER.

(From the Division of Soils, Minnesota Agricultural Experiment Station, St. Paul.)

(Received for publication, June 9, 1916.)

INTRODUCTION.

In a recent article (Gortner and Blish, 1915) the statement was made that "in all probability the humin nitrogen of protein hydrolysis has its origin in the tryptophane nucleus." This statement was based upon the following findings.

1. Zein, which contains no tryptophane, yields but a very small amount of humin nitrogen on hydrolysis (compare also Osborne and Jones, 1910).

2. *When tryptophane was added to zein and the mixture hydrolyzed, a very considerable quantity of humin nitrogen was produced.*

3. This humin was formed from tryptophane *plus* some other product of protein hydrolysis, inasmuch as the formation of humin from 1 gm. of zein plus tryptophane had reached a maximum where 0.125 gm. of tryptophane had been added, and the addition of tryptophane in excess of this amount failed to increase the humin nitrogen.

4. When tryptophane was heated with hydrochloric acid in the presence of a carbohydrate 86.56 per cent of the tryptophane nitrogen was recovered in the humin, and we suggested that perhaps the reaction involved in the humin formation was the condensation of an aldehyde with the $-NH$ group of the tryptophane nucleus (compare also Homer, 1913, a).

5. We suggested that it might be possible so to alter the conditions of hydrolysis as to make the tryptophane plus carbohydrate reaction a quantitative method for the determination of

tryptophane, and stated that we did not have sufficient material at that time to work out the ideal conditions of the reaction.

6. We found that the adsorption of ammonia by the non-nitrogenous humin formed from carbohydrates could not be an important factor in the formation of humin nitrogen.

These findings were later confirmed in part and accepted by Osborne, Van Slyke, Leavenworth, and Vinograd (1915), and by Van Slyke (1915), these authors accepting the humin nitrogen formed in the presence of carbohydrate as representing 86 per cent of the tryptophane nitrogen present in the original protein. Later, Grindley and Slater (1915) took strong exception to our findings and state: "Unfortunately for the good of the methods for the analysis of proteins the conclusion of Gortner and Blish that in all probability the humin nitrogen of protein hydrolysis has its origin in the tryptophane nucleus is apparently not true, since humin contains in addition adsorbed nitrogen from other amino-acids."

It seems to me that this is a rather sweeping statement to make in the face of findings (1) and (2) noted above. Had Grindley and Slater expressed doubts as to the humin nitrogen being a *quantitative* measure of the tryptophane content of the protein, or that other humin formed in the presence of carbohydrate was a quantitative measure of tryptophane, I should probably agree, inasmuch as we stated definitely that our data did not permit us to state the conditions under which a quantitative conversion of tryptophane to humin could be obtained. It is to be regretted if our statement, "When an abundance of carbohydrate is present nearly 90 per cent of the tryptophane nitrogen remains in the humin nitrogen fraction. It is suggested that this property be utilized to determine the approximate quantity of tryptophane in proteins," conveyed the idea that we put this forward as a quantitative method. What we intended to suggest was that some one should utilize this property to elaborate a method which would be quantitative. Inasmuch as no one has attempted to do this, I have again taken up the problem and, as will be shown below, have found that carbohydrates cannot be used. However, I still believe (due to observations (1) and (2) above) that our original conclusion is very near the truth; *i.e.*, "in all probability the humin nitrogen of protein

hydrolysis *has its origin* (at least in part) in the tryptophane nucleus." In making this statement we were thinking of the hydrolysis of a *pure protein* to which nothing had been added.

At this point it may be well to consider the material with which Grindley and Slater worked, the results from which apparently caused them to question our findings. They subjected blood meal, tankage, alfalfa hay, white soy beans, cottonseed meal, oats, barley, rolled wheat, and whole wheat to acid hydrolysis and *proceeded with the analysis of the resulting hydrolysate, using Van Slyke's method, in exactly the same manner as though they were dealing with a pure protein.* They express the results obtained from the basic fraction as "arginine N," "cystine N," "histidine N," and "lysine N." What justification is there for this? Indeed what justification is there for their term "humin nitrogen" if by humin nitrogen we mean the nitrogen originating from protein material by acid hydrolysis? Van Slyke (1915) recently called attention to the fact that his methods were devised to be applied to pure protein material and not to a heterogeneous mixture of nitrogen compounds. To be sure many valuable *comparative* data can be obtained by the application of Van Slyke's methods to heterogeneous material, and I have already (1913) used them in this connection, being careful to state that the "arginine," "histidine," etc. were not necessarily pure arginine and histidine, but that the nitrogen of these fractions reacted in the same manner as arginine or histidine nitrogen of a protein, and that the results, while not *exact*, were *comparative* when compared with similar material.

In this connection it is of interest to note the findings of Janney (1916) on the protein content of muscle. He states that approximately *13 per cent of the nitrogen of muscle is combined in non-protein substances.*

If muscle contains such a large percentage of non-protein nitrogen, it seems very probable that in the material analyzed by Grindley and Slater, there must have been many nitrogenous organic compounds which had no relation to the protein molecule, such as nitrogenous fats, nitrogenous pigments, purine bases, pyrimidine bases, etc. How will these behave to acid hydrolysis? How will they be distributed in the subsequent distribution by Van Slyke's method? For *comparative* results

these questions need not be answered, but it is self-evident that *no direct analogy can be drawn between the analysis of a protein mixed with an unknown amount of these nitrogenous compounds and the analysis of pure proteins.* It is certain that the humin nitrogen will be altered to a marked extent by the presence of many of these compounds; the humin nitrogen of such a mixture represents the sum total of all the nitrogenous compounds which are insoluble in hot water or dilute calcium hydroxide, together with calcium salts of nitrogenous organic acids or the purine or pyrimidine bases in addition to the humin formed from the protein present. The humin nitrogen (from protein material) actually present in such a mixture may easily be a very small part of the nitrogen found. This is equally true of the "humin nitrogen" reported by Potter and Snyder (1915) for the hydrolysate of certain soils and soil extracts. In one instance the "humin nitrogen" attains to 21.5 per cent of the total nitrogen. It is perfectly obvious that while Potter and Snyder's results are extremely valuable as *comparative* results, they do not give us exact data regarding the nature of the proteins present in the soil. The actual arginine, histidine, and lysine present in the soil would probably differ widely from the quantities indicated by the method of analysis *because this method gives accurate data regarding amino-acids only when applied to the analysis of pure protein.*

I have shown (1913) that uric acid nitrogen is distributed after acid hydrolysis 15.3 per cent as ammonia nitrogen, 35.98 per cent as humin nitrogen, 12.97 per cent as basic nitrogen, and 35.78 per cent in the filtrate from the bases. The "humin nitrogen" in this instance contained no trace of black color and was probably calcium urate.

In this paper as well as in the previous one I am concerned only with the problem of *protein nitrogen* and I am ready to grant that in all probability the results already given, and those which will be given below, will not apply to the analysis of heterogenous mixtures of nitrogenous compounds.

EXPERIMENTAL.

Throughout this work only one protein has been used. The protein selected was Merck's "fibrin from blood." This selection was made, partly because of the ease with which the material could be secured, and partly because analyses were already on record (Van Slyke, 1911) with which comparison could be made. A protein high in lysine was purposely chosen for reasons which will be shown later.

The tryptophane represented a part of the material, the analysis of which was reported in our previous paper (Gortner and Blish, 1915). The tyrosine was prepared from silk waste in the usual manner (Hoppe-Seyler, 1909) and was repeatedly recrystallized from hot water.

A. Hydrolysis of Protein in the Presence of Carbohydrates.

As far as I am aware, the only data dealing with the hydrolysis of a pure protein in the presence of carbohydrate material are those which have been reported from this laboratory (Gortner and Blish, 1915) and those reported in confirmation of these results by Osborne (1915). A summary of these data is given in Tables I and II.

TABLE I.

The Effect of Added Dextrose on the Hydrolysis of a Pure Protein (Data of Gortner and Blish, 1915).

Material hydrolyzed.	Nitrogen content.	Ammonia N.	Humin N.	Basic N.	Non-basic N.
gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Zein 1	0.1525	0.0316	0.0007	0.00489	0.1184
Zein 1 + tryptophane 0.125	0.1696	0.0316	0.0077	0.0056	0.1241
" 1 + " 0.25	0.1868	0.0290	0.0070	0.0160	0.1348
" 0.5 + dextrose 0.5	0.0762	0.0154	0.0014		
" 0.5 + tryptophane 0.125 + dextrose 0.5	0.0933	0.0158	0.0154		
Tryptophane 0.0125 + dextrose 0.5	0.0171	None.	0.0148		
Gliadin 1	0.1700	0.0451	0.0010	0.0071	
" 1 + dextrose 0.25	0.1700	0.0454	0.0016	0.0068	
" 1 + " 2	0.1700	0.0450	0.0039	0.0063	

TABLE II.

The Effect of Added Dextrose on the Estimation of the Amino-Acids, by Van Slyke's Method (Data of Osborne, 1915).

Nitrogen.	Lactalbumin hydrolyzed alone.	Lactalbumin + equal weight of dextrose.
	per cent	*per cent*
Ammonia	8.56	8.37
Humin	2.32	3.70
Cystine	1.30	1.05
Arginine	7.20	8.10
Histidine	4.57	3.22
Lysine	12.24	12.54
Amino N, in filtrate	62.0	58.79
Non-amino, in filtrate	2.0	3.58
Total	100.19	99.35

The Hydrolysis of Fibrin in the Presence of Equal Amounts of Different Carbohydrates.—3 gm. of fibrin were boiled for 48 hours with an equal amount of dextrose, lactose, levulose, sucrose, maltose, cellulose (Swedish filter paper), and soluble starch (Merck's) in the presence of 75 cc. of 20 per cent HCl. Ammonia nitrogen and humin nitrogen were determined according to Van Slyke (1911), using the entire hydrolysate. The data are given in Table III.

It will be observed that, in every instance, the added carbohydrate approximately doubled the humin nitrogen while the ammonia figures are only slightly altered. It is probable that there is a very slight loss of ammonia nitrogen due to the added carbohydrate but this loss is practically negligible in a protein analysis. The 3 gm. of fibrin taken for analysis contained approximately 0.4550 gm. of nitrogen so that the maximum change of 0.00352 gm. ammonia nitrogen would equal only 0.77 per cent of the total nitrogen. That the actual change in the amount of ammonia nitrogen is considerably less than this is shown by a reference to Table IV where, in two duplicate determinations, 9 gm. of carbohydrate altered the ammonia nitrogen only 0.69 and 0.16 per cent of the total nitrogen. The last figure is well within the maximum error allowed by Van Slyke (1911), 0.37 per cent. It seems very probable, however, that the lower

TABLE III.

The Ammonia and Humin Nitrogen Obtained from 3 Gm. of Fibrin Hydrolyzed in the Presence of Equal Amounts of Different Carbohydrates.

Carbohydrate added (3 gm.).	Ammonia N.	Increase (+) or decrease (−).	Humin N.	Increase (+) or decrease (−).
	mg.		*mg.*	
None	46.35		13.15	
	46.55		13.35	
	46.15		13.15	
	46.85		12.85	
	44.20*		†	
	46.20		†	
Average	46.42		13.13	
Soluble starch	42.90	−3.52	28.60	+15.47
" "	43.05	−3.37	28.60	+15.47
Dextrose	44.65	−1.77	24.75	+11.62
Sucrose	43.50	−2.92	24.30	+11.17
Maltose	43.90	−2.52	25.40	+12.27
Levulose	43.15	−3.27	24.15	+11.02
Lactose	44.20	−2.22	26.75	+13.62
Cellulose (Swedish filter paper)	45.90	−0.52	23.80	+10.67
" " " "	46.90	+0.48	25.30	+12.17

* Omitted from average.

† Humin not determined in the usual manner on these solutions.

ammonia determination is due to a slower rate of hydrolysis when carbohydrates are present. Osborne (1915) states that hydrolysis is slower in the presence of carbohydrate material. A more rapid hydrolysis would have the same effect as a longer hydrolysis, and more ammonia N would be formed (compare Table VIII and footnote 2).

The increase in the humin nitrogen does not appear to be constant, but I believe that most of the variation is caused by unavoidable errors in the washing of the humin. In some instances the humin "balled" together, and it is possible that channels were formed so that the washing was incomplete. In some instances the filter practically clogged with a gummy humin, while in other instances the humin was granular and washed as fast as water could be poured through it. It was found near the end of the experiments that the amount of calcium hydroxide added in the ammonia determination determined, to a great

extent, the nature of the humin. In the presence of a large excess of lime the humin was usually gummy and filtered slowly, while the granular condition was obtained when the lime was present in only a slight excess. The table does show, however, that the ammonia nitrogen is not greatly changed by the added carbohydrate and that there is a fairly uniform increase in humin nitrogen regardless of the nature of the carbohydrate added.

Hydrolysis of Fibrin in the Presence of Increasing Amounts of Carbohydrate.—3 gm. of fibrin were hydrolyzed in the presence of 1.5, 3.0, 6.0, and 9.0 gm. of cellulose (Swedish filter paper) by boiling with 75 cc. of 20 per cent HCl for 48 hours. The ammonia and humin nitrogen data are shown in Table IV.

TABLE IV.

The Ammonia and Humin Nitrogen Obtained from 3 Gm. of Fibrin Hydrolyzed in the Presence of Increasing Amounts of Carbohydrate.

Swedish filter paper added.	Ammonia N.	Increase (+) or decrease (−).	Average change.	Humin N.	Increase (+).	Average change.	Average increase due to each 1.5 gm. carbohydrate added.
gm.	*mg.*		*per cent*	*mg.*		*per cent*	*per cent*
None(average)	46.42			13.13			
1.50	46.70	+0.28	+0.60	19.95	+ 6.82	51.94	51.94
3.0	46.90	+0.48		24.65	+11.52		
	45.90	−0.52	−0.09	23.80	+10.67	84.53	32.59
6.0	44.80	−1.62		29.35	+16.22		
	45.00	−1.42	−3.27	30.55	+17.32	127.7	21.59
9.0	43.30	−3.12		33.55	+20.42		
	45.70	−0.72	−4.14	34.85	+21.72	160.4	16.37

It will be observed that there is no uniform increase or decrease of the ammonia nitrogen due to the added carbohydrate, the figures obtained suggesting only chance variation.

The humin nitrogen, however, shows a continuous increase, but *this increase is not a direct function of the added carbohydrate* but seems to result from two or more reactions. On the addition of only 1.5 gm. of cellulose the humin nitrogen increased almost 52 per cent. This is nearly twice the increase due to the second 1.5 gm. (32.59 per cent). The increase in humin nitrogen continues to fall until there is average increase of only

16.37 per cent for the fifth and sixth 1.5 gm. of carbohydrate added.

If the mg. of humin nitrogen obtained are plotted against the gm. of added carbohydrate it will be found that a very regular curve is obtained, rising quite rapidly at first and then flattening out, with no indication of ever reaching a straight line maximum. This experiment seems to confirm Grindley and Slater (1915) in their statement that adsorption of amino-acids by the humin formed from carbohydrates must be taken into account. Adsorption, absorption, or occlusion must be the cause of at least a part of the continued increase. These factors, however, cannot explain the rapid increase of the humin nitrogen due to the first addition of carbohydrate, where the reaction is undoubtedly largely a chemical one.

Comparative Analyses of Fibrin Hydrolyzed Alone and in the Presence of Three Times Its Weight of Cellulose.—As noted earlier, Osborne (1915) hydrolyzed lactalbumin in the presence of an equal weight of dextrose and found that, aside from an increase in the humin nitrogen, there was no notable change in any of the other fractions. As regards the basic fractions they state: "The total nitrogen of the bases was not reduced at all nor are the proportions of amino to non-amino nitrogen in the bases significantly affected." Their analyses are reprinted in Table II.

Grindley and Slater (1915), on the other hand, state that when lysine and cystine are heated in the presence of carbohydrates (quantity not given) 4.7 per cent of the lysine nitrogen and 6.3 per cent of the cystine nitrogen remain in the humin fractions. The loss of 6.3 per cent of the cystine nitrogen would in all Van Slyke analyses be entirely negligible, excepting in the analysis of keratin material. This is shown by reference to Table V, the cystine content of the protein being taken from Van Slyke's (1911) data.

As to the adsorption of 4.7 per cent of the lysine N by the humin, Osborne (1915) showed that when an *equal* weight of carbohydrate was added to a protein no such adsorption was detectable. They find f om 12.09 to 12.35 per cent of lysine in lactalbumin when hydrolyzed alone and 12.54 per cent in lactalbumin hydrolyzed in the presence of an equal weight of dextrose. *If 4.7 per cent of the lysine had been adsorbed by the humin the latter figure should*

TABLE V.

The Error Introduced into a Van Slyke Analysis Due to the Adsorption of 6.3 Per Cent of the Cystine Nitrogen by the Humin.

	Cystine content (Van Slyke's (1911) data).	Humin N gain due to 6.3 per cent of cystine.
	per cent	*per cent*
Gliadin	1.25	0.078
Edestin	1.49	0.093
Dog hair	6.60	0.496
Fibrin	0.99	0.062
Hemocyanin	0.80	0.020

have been only 11.67 per cent. In any case, however, the differences are within the 0.63 per cent given (by Van Slyke, 1911) as the "average difference" between duplicate determinations.

It seemed well, however, to make a complete analysis of a protein hydrolyzed in the presence of a large quantity of carbohydrate. Two separate portions of approximately 3 gm. of fibrin and 9 gm. of Swedish filter paper were hydrolyzed on an electric hot plate with 100 cc. of 1.115 sp. gr. HCl for 48 hours, and at the same time two flasks containing an equal amount of fibrin and acid but no carbohydrate were hydrolyzed on the same hot plate. The resulting solutions were analyzed according to Van Slyke (1911, 1912, a) using the minor modifications (as regards dilution, etc.) that I have reported previously (1913).[1] The bases were precipitated from a volume of 200 cc., were filtered off after cooling to nearly 0°, and washed with washing solution also cooled to near 0°. The figures are not corrected for the solubility of the basic phosphotungstates. The comparative analyses are shown in Table VI.

It will be seen that the most notable change occurs in the humin nitrogen. Table VII shows the differences between the duplicate determinations, the differences apparently due to the addition of the 9 gm. of carbohydrate to 3 gm. of fibrin, as well as the differences between lactalbumin hydrolyzed alone and in the presence of an equal weight of dextrose (data of Osborne, 1915). Van Slyke's (1911) "maximum difference" and "aver-

[1] In making these analyses I overlooked the recent modification of Van Slyke (1915) and liberated the bases with baryta as in the earlier directions.

TABLE VI.

Comparative Analyses of 3 Gm. Fibrin Hydrolyzed Alone and in the Presence of 9 Gm. of Carbohydrate Material.

Nitrogen.	Nitrogen.		Per cent of total nitrogen.		
3 gm. fibrin, no carbohydrate.	I.	II.	I.	II.	Average.
	gm.	*gm.*			
Total	0.4551	0.4618			
Ammonia	0.04615	0.04685	10.14	10.15	10.15
Humin	0.01315	0.01285	2.89	2.78	2.83
Basic	0.1248	0.1304			
Arginine	0.0480	0.0520	10.54	11.27	10.91
Hystidine	0.0197	0.0203	4.33	4.40	4.36
Lysine	0.0550	0.0555	12.09	12.01	12.05
Cystine	0.0021	0.0026	0.46	0.56	0.51
Amino, in bases	0.0757	0.0779			
Amino, in filtrate from bases	0.2571	0.2510	56.49	54.37	55.43
Non-amino, in filtrate from bases	0.0108	0.0122	2.37	2.64	2.51
Total recovered	0.4520	0.4533	99.32	98.18	98.75
3 gm. fibrin + 9 gm. cellulose.					
Total	0.4505	0.4528			
Ammonia	0.0433	0.0457	9.61	10.09	9.85
Humin	0.0342	0.0355	7.59	7.84	7.72
Basic	0.1166	0.1107			
Arginine	0.0405	0.0368	8.99	8.13	8.56
Hystidine	0.0234	0.0205	5.19	4.53	4.86
Lysine	0.0495	0.0502	10.98	11.09	11.04
Cystine	0.0032	0.0032	0.71	0.71	0.71
Amino, in bases	0.0706	0.0694			
Amino, in filtrate from bases	0.2295	0.2404	50.94	53.09	52.02
Non-Amino, in filtrate from bases	0.0185	0.0168	4.11	3.71	3.91
Total recovered	0.4421	0.4491	98.13	99.19	98.67

age difference" to be expected between duplicate determinations are also included in the table for reference.

By a study of Table VII it will be seen that the differences between the analyses of those proteins hydrolyzed alone and in the presence of carbohydrate material not to exceed three times

the weight of the protein are, in the case of most of the fractions, within the maximum allowed by Van Slyke for experimental error. The only differences which are *certainly* greater than experimental error are those of humin nitrogen and amino nitrogen in the filtrate from the bases. It seems probable, however, that there is a significant loss in the arginine N and a gain in the non-amino N in the filtrate from the bases. As regards the other observed changes, I am inclined to regard them as due to experimental error, although the change in lysine N due to hydrolysis in the presence of three times the protein's weight of carbohydrate may be significant.

The data already given in Tables IV, VI, and VII indicate that an exact determination of the chemical groups in proteins

TABLE VII.

The Difference between Duplicate Analyses (Due to Experimental Errors), the Differences Apparently Due to the Addition of Carbohydrate, as Well as Van Slyke's "Maximum" and "Average" Differences to Be Expected.

Nitrogen.	Difference between duplicates of fibrin alone.	Difference between duplicates of fibrin and 3 times its weight of carbohydrate.	Average difference between fibrin hydrolyzed alone and with 3 times its weight of carbohydrate.	Difference between lactalbumin hydrolyzed alone and with an equal weight of dextrose.*	Van Slyke's (1911) experimental differences.	
					"Maximum."	"Average."
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Ammonia	0.01	0.48	−0.30	−0.19	0.37	0.12
Humin	0.11	0.25	+4.89	+1.38	0.39	0.20
Arginine	0.73	0.86	−2.35	+0.90	1.27	0.73
Hystidine	0.07	0.66	+0.50	−1.35	2.14 (0.93)†	0.79
Lysine	0.08	0.11	−1.01	+0.30	1.23	0.61
Cystine	0.10	0.00	+0.20	−0.25	0.11	0.05
Amino, in filtrate from bases	2.12	2.15	−3.41	−3.21	1.60 (0.60)	0.63
Non-amino, in filtrate from bases	0.27	0.40	+1.40	+1.58	1.20	0.68

* Data of Osborne (1915).

† The figure in brackets represents the second greatest difference between duplicates observed by Van Slyke (1911).

by Van Slyke's method cannot be expected when an amount of carbohydrate equal to three times the weight of the protein is present. To make this point doubly sure, however, the following experiment was carried out.

The filtrates from the humin determinations of 3 gm. of fibrin, hydrolyzed alone and in the presence of 3 gm. of soluble starch were evaporated to approximately 35 cc. volume *in vacuo*, a large excess of concentrated hydrochloric acid was added, and the solution again evaporated *in vacuo* to about 35 cc. This solution was washed out into the hydrolyzing flasks with 1.115 sp. gr. HCl so that the total volume was 75 to 100 cc. 3 gm. of soluble starch were added to the residue, which had been previously hydrolyzed in the presence of starch, and nothing was added to the residue which had been hydrolyzed alone. The contents of the flasks were boiled again for 48 hours, and ammonia N and humin N determined on the resulting solutions as usual. The data are given in Table VIII. It will be seen that there is a marked gain of ammonia nitrogen which does not differ greatly in the case of the fibrin + carbohydrate from that of the fibrin alone. The differences are believed to be within experimental error. Van Slyke (1912, b) has observed this increase of ammonia

TABLE VIII.

The Ammonia N and Humin N Formed from the Filtrates from the Humin of 3 Gm. of Fibrin + 3 Gm. of Starch when Boiled a Second Time in the Presence of 3 Gm. of Starch, Compared with a Second Boiling (without Carbohydrate Addition) of the Filtrate from the Humin of 3 Gm. of Fibrin Hydrolyzed Alone.

	Ammonia N.	Average per cent of total N.	Humin N.	Average per cent of total N.
	mg.		*mg.*	
3 gm. fibrin + 3 gm. starch (1st hydrolysis)	42.90		28.60	
	43.05	9.45	28.60	6.29
Filtrate from humin of above hydrolysate + 3 gm. starch (2nd hydrolysis)	7.80		5.85	
	8.05	1.74	6.35	1.34
3 gm. fibrin, no carbohydrate (1st hydrolysis)	46.35		13.15	
	46.55	10.02	13.35	2.91
Filtrate from humin of above hydrolysate, no carbohydrate (2nd hydrolysis)	10.00		0.65	
	8.15	2.00	1.15	0.20

N due to prolonged hydrolysis and in certain unpublished work carried out before Van Slyke's results were published I made a similar observation.[2] In Table VIII there is an increase of 18.30 per cent of the original ammonia nitrogen due to a further hydrolysis. The origin of this ammonia, and the length of time the increase will continue are now being studied at this Experiment Station.

The humin nitrogen increases only 6.79 per cent of the original humin N on further hydrolysis when no carbohydrate is added, and 21.32 per cent on the addition of more carbohydrate (actually 46.04 per cent increase calculated on the original fibrin + no carbohydrate). This would indicate that the increase of 115.8 per cent of humin N in the first hydrolysis was caused by a chemical reaction, augmented by adsorption or occlusion of other amino-acids by the carbohydrate humin. The further increase of 46.04 per cent of the humin nitrogen on a second hydrolysis is almost conclusive proof of such adsorption, absorption, or occlusion.

A Comparison of Van Slyke's (1911) Analysis of Fibrin with the Data of Table VI.—When my analysis of fibrin was compared

[2] The following results were obtained in February and March, 1911, at the Station for Experimental Evolution, Carnegie Institution of Washington.

Kahlbaum's casein *nach Hammarsten* (not further purified or dried):

1 gm.	hydrolyzed	8	hrs.	gave	0.0143	gm.	NH_3	N.
1 "	"	26	"	"	0.0153	"	"	"
1 "	"	120	"	"	0.0183	"	"	"

or 19.5 per cent increase over the 26 hours' hydrolysis.

Fibrin:

1 gm.	hydrolyzed	7	hrs.	gave	0.0131	gm.	NH_3	N.
1 "	"	25	"	"	0.0144	"	"	"
1 "	"	90	"	"	0.0179	"	"	"
1 "	"	115	"	"	0.0195	"	"	"

the last figure being 35.4 per cent increase over 25 hours' hydrolysis.

Egg albumin:

1 gm.	hydrolyzed	6.5	hrs.	gave	0.0112	gm.	NH_3	N.
1 "	"	48	"	"	0.0146	"	"	"
1 "	"	96	"	"	0.0168	"	"	"

the last figure being 15.0 per cent increase over 48 hours' hydrolysis.

Van Slyke's results were announced before the work could be repeated and confirmed on other proteins, and inasmuch as his data were in substantial agreement with my own the investigation was discontinued.

with the analysis of Van Slyke (1911), as in Table IX, there is observed a very close agreement in all of the figures excepting in the ammonia N and arginine N. The difference in the ammonia figures is undoubtedly due to the fact that Van Slyke hydrolyzed his fibrin for a shorter period than 48 hours.[3]

TABLE IX.

A Comparison of Fibrin Analyses by Van Slyke and by the Author.

Nitrogen.	Van Slyke.*	Gortner.
	per cent	*per cent*
Ammonia	8.32	10.15
Humin	3.17	2.83
Cystine	0.43	0.51
Arginine	13.14	10.91
Hystidine	3.96	4.36
Lysine	11.40	12.05
Amino, in filtrate	55.40	55.43
Non-amino, in filtrate	3.85	2.51
Total	99.67	98.75

* Van Slyke's figures are those which were not corrected for solubility of the bases. These were taken, instead of the corrected values, so that they would be directly comparable with my data.

B. Hydrolysis of Proteins in the Presence of Aldehydes.

In the preceding paper (Gortner and Blish, 1915) the following statement was made:

"When carbohydrates are boiled with mineral acids, a small amount of furfural is formed and it seems highly probable that the reaction involved in humin formation is the condensation of tryptophane with an aldehyde. Miss Homer (1913, a) has prepared condensation products of tryptophane with various aldehydes and states: 'Indole derivatives by virtue of the —NH group in the nucleus will react with formaldehyde and trioxymethylene *in the presence of a condensing agent* to form substances of intense color and marked insolubility in ordinary solvents other than concentrated mineral acids.' "

It is well known that aldehydes produce certain color reactions when added to protein material, and that addition products and condensation

[3] Van Slyke does not mention the length of hydrolysis for fibrin but in other data in the same paper he speaks of hydrolyzing for 28 hours.

products can be obtained between aldehydes and certain of the amino-acids. For example, the Adamkiewicz reaction or glyoxylic reaction (Hopkins and Cole, 1901) of proteins appears to be due to the presence of nascent formaldehyde acting upon tryptophane.

Reichl's (1890–91) test for tryptophane in proteins depends upon a color reaction produced in the presence of benzaldehyde, 50 per cent sulfuric acid, and some oxidizing agent (ferric chloride).

The Ehrlich-Neubauer (1903) and Rohde (1905) test for tryptophane is dependent upon a coloration produced by *p*-dimethylaminobenzaldehyde, and Herzfeld (1913) has utilized this coloration to elaborate a colorimetric method for the estimation of tryptophane in the tryptic digest of proteins.

Rohde (1905) finds that the aliphatic aldehydes, formic, acetic, propionic, or butyric, do not give color reactions with proteins, that furfural or citral give only a yellowish color, while all aromatic aldehydes produce more or less brilliant colors, varying from red to blue or green, depending upon the aromatic aldehyde used. He, likewise, finds that gelatin, carefully purified, produces no color with aromatic aldehydes, and that tyrosine + gelatin does not give a color. He therefore concludes that the color is produced only in the presence of tryptophane.

Steensma (1906) extends Rohde's work by testing the reaction of other aldehydes and by showing the color changes which may be induced by a trace of an oxidizing agent (nitrous acid).

It would seem that all of the color tests referred to above are produced by reactions which involve the $-NH$ group of tryptophane instead of the aliphatic amino group. Miss Homer (1913, a and b, and 1915) finds that, when a condensing agent is present, formaldehyde and trioxymethylene react with tryptophane to form "substances of intense color and marked insolubility in ordinary solvents." Of course this is merely the Adamkiewicz reaction, or the glyoxylic reaction, carried further, so that the colored product is actually isolated.

The aliphatic amino group, likewise, reacts readily with aldehydes. Miss Homer (1913, a) prepared a number of condensation products of indole derivations with aldehydes, tryptophane being one of the indole compounds studied. She was able to isolate a colorless, crystalline compound of tryptophane and formaldehyde, the condensation taking place on the aliphatic amino group of the tryptophane. It seems very probable that similar crystalline condensation products could be prepared from all, or almost all, of the amino-acids, not only with formaldehyde, but with other aldehydes. That such a reaction takes place very readily between amino-acids and formaldehyde in neutral

solution is evinced by the well known method of Sörensen (1908), where formaldehyde is used to titrate the free amino-acids in a solution, the reaction taking place being

$$R\,CH(NH_2)COOH + HCHO = R\,CH(N:CH_2)COOH + H_2O.$$

Trillat (1892), Blum (1896), Benedicenti (1897), Schwarz (1900), and Treves and Salomone (1908) studied a similar type of reaction in which the amino-acids are replaced by an entire protein molecule. They find that a chemical combination takes place between aldehydes and proteins, presumably on the aliphatic amino group, to form aldehyde-proteins. These compounds possess properties which are widely different from the parent protein; for example, aldehyde-albumin formed from egg-white is not coagulated on boiling and is not attacked by trypsin. It is claimed that all of the aldehyde (formaldehyde) can be removed from the aldehyde-protein by steam distillation. If this is actually the case, it would indicate a somewhat loose combination, and would also indicate that the $-NH$ group of tryptophane and the hydroxyl group of tyrosine were not involved in the reaction. Additional data are desirable on this point.

Ultimate analyses of aldehyde-proteins (Schwarz, 1900) and of indole and skatole condensation products with trioxymethylene (Homer 1913, a) are on record, but I have been unable to find any literature bearing on the subject of the *nitrogen distribution* either in pure proteins or with individual amino-acids, after heating with aldehydes in the presence of a condensing agent. It seemed advisable, therefore, to make some experiments in which proteins were hydrolyzed in the presence of aldehydes.

The results which will be reported in the following pages are unavoidably incomplete,[4] and serve mainly to show that there is here a field worthy of more complete investigation. The data which have been collected to date are here presented, and active work in this field will be continued at once. Unless some one has already begun a study of the effect of the presence of aldehydes on the nitrogen distribution in the hydrolysates of proteins and

[4] On Aug. 1, 1916, the writer transferred from the Division of Soils to the Division of Agricultural Bio-Chemistry, in charge of the Section of Biochemical Research.

protein materials, I should like to reserve this field for the present.

Hydrolysis of Fibrin in the Presence of Furfural.—As is noted above, Gortner and Blish (1915) postulated that perhaps the reactivity of the carbohydrate was due to furfural. The following experiments were therefore made.

3 gm. of fibrin were hydrolyzed by boiling for 48 hours with 75 cc. 1.115 sp. gr. HCl in the presence of 0.25, 0.50, 1.0, and 5.0 cc. of freshly distilled furfural.

On adding the furfural to the HCl + fibrin, an intense red-violet coloration formed at once in the cold. The flakes of fibrin rapidly darkened in the cold solution so that within 2 or 3 minutes they were jet-black.

On an attempt to heat the mixture of 5 cc. of furfural + 75 cc. HCl + 3 gm. of fibrin a vigorous reaction took place as soon as the solution began to get warm, followed almost at once by a violent explosion which shattered the flask. The reaction began in much the same manner that sucrose and concentrated sulfuric acid react to form charcoal.

In the other experiments where smaller quantities of furfural were used, successful hydrolysis was obtained, although the large amount of humin caused trouble in several instances in the flasks containing 1 cc. of furfural. In two instances hydrolysates containing 1 cc. of furfural were lost because of minor explosions, throwing a part of the contents out of the flasks. The humin in all instances was jet-black and granular, washed quite readily, and was very bulky, that from the hydrolysate containing 1 cc. of furfural almost filling a 15 cm. filter paper. In all experiments, except that where only 0.25 cc. of furfural was used, the hydrolysate was clear, excepting for the suspended solid mass of humin; *i.e., all color was insoluble.* The experimental data are shown in Table X.

It will be seen that we have here much the same type of reaction as that shown by varying the amount of carbohydrate (Table IV); *i.e., a sudden initial rise in the humin nitrogen, followed by a slow increase which shows no sign of soon reaching a maximum.* This would indicate a chemical combination followed by adsorption or occlusion. Indeed, it seems to me to

TABLE X.

The Effect on Ammonia N and Humin N of Hydrolyzing 3 Gm. of Fibrin in the Presence of Varying Amounts of Furfural.

Furfural added.	Ammonia N.	Increase (+) or decrease (−).	Average change.	Humin N.	Increase (+).	Average change.	Average increase due to each 0.25 gm. furfural.
cc.	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*per cent*
None (average).	46.42			13.13			
0.25	45.20	−1.22	−2.63	20.20	+ 7.07	+53.84	53.84
0.50	43.90	−2.52	−5.43	22.05	+ 8.92	+67.94	14.09
1.0	44.15	−2.27	−4.89	26.05			
1.0	Lost.			26.55	+13.17	+100.3	16.18

be extremely probable that the humin formed from carbohydrates is actually formed from furfural, which is in turn formed from carbohydrates. The polymerization (?) of furfural to humin by strong HCl takes place very rapidly, and, when a very considerable amount of furfural is present, may assume explosive violence.

In order to arrive at some idea as to the proportions of humin formed from furfural, and from carbohydrate material, I boiled 1 cc. of furfural (1.165 gm.) with 100 cc. of 1.115 sp. gr. HCl for 18 hours, filtered the resulting humin on a weighed Gooch crucible, washed with warm water, dried at 105°, and weighed; 0.8902 gm. of black insoluble "humin" was obtained or 76.40 per cent of the original furfural. From 3 gm. of Swedish filter paper treated in a similar manner only 0.4650 gm. humin was obtained or 15.50 per cent of the original carbohydrate.

It is obvious from the above work that carbohydrates and furfural behave in the same manner when added in a protein hydrolysis. Both chemical and physical reactions take place and the increase in the nitrogen content of the humin cannot be definitely assigned to any amino-acid or groups of amino-acids.

Hydrolysis of Fibrin in the Presence of Benzaldehyde.—The preceding experiment was repeated, replacing the furfural with benzaldehyde using 0.5, 1.0, 2.0, and 4.0 cc., respectively. Benzoic acid crystals were present in all flasks at the end of the hydrolysis. Free benzaldehyde likewise distilled off in all experiments, except that where 0.5 cc. was added, and in this instance there was still a detectable odor.

In the case of 1, 2, and 4 cc. of benzaldehyde, the humin "balled" together into a cake which was pasty when hot, but hard when cold. The liquid in the flasks had no trace of black color, except in that where only 0.5 cc. of benzaldehyde was added. In all of the others the liquid was light yellow during the last half of the hydrolysis. This is a notable exception to the usual course of hydrolysis (compare also the furfural, which acted in a similar manner).

After the first experiments had been completed, it was found desirable to separate the humin into two fractions, "acid-insoluble" and "acid-soluble." The acid-insoluble humin was obtained in two additional experiments by diluting the hydrolysate, filtering off the insoluble humin, and washing it free from acid. The filtrate from the acid-insoluble humin was then concentrated as usual, to get rid of the excess of HCl, calcium hydroxide added, and the ammonia distilled off. The "acid-soluble" humin was adsorbed by or combined with the lime, and was determined in the usual manner for the humin determination.

In those experiments where no benzaldehyde was added, the filtrate from the "acid-insoluble" humin was jet-black, and, as is shown in the table, contained nearly 50 per cent of the normal humin N. The "acid-soluble" humin from the benzaldehyde experiments formed a light tan colored precipitate, very small in amount as compared with the usual humin precipitate. The data are shown in Table XI.

It will be noted that decidedly different results are obtained with benzaldehyde from those obtained in the presence of carbohydrates or furfural. The reactions here appear to be wholly chemical. The ammonia nitrogen is probably not significantly altered by the amounts of benzaldehyde used, although, as will be shown later, there is possibly some deamination with the larger quantities of benzaldehyde.

The humin nitrogen rises rapidly to a maximum of approximately double the amount of the humin N produced in the ordinary hydrolysis. There is some indication that the maximum amount of humin nitrogen is not reached when the larger quantities of benzaldehyde are present. This is almost certainly the case when the benzaldehyde is replaced by formaldehyde (see later). The major part (92 to 95 per cent) of the humin nitrogen formed

TABLE XI.

The Effect on Ammonia N and Humin N of Hydrolyzing 3 Gm. of Fibrin in the Presence of Varying Amounts of Benzaldehyde.

Benzaldehyde added.	Ammonia N.	Change.	Acid-insoluble humin.	Acid-soluble humin.	Total humin.	Increase.	
cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
None (average).	46.42				13.13		
0.5	46.05	−0.37			23.15	10.02	76.30
1.0	44.75	−1.67			25.75	12.62	96.12
2.0	48.70	+2.28			25.80	12.67	96.49
4.0	52.65	+6.23			25.25	12.12	92.30
1.0	46.05	−0.37	23.70	2.05	25.75	12.62	96.12
3.0	47.50	+1.08	23.45	0.85	24.30	11.17	85.04
None.	44.20		6.50	5.55	12.05		
"	46.20		7.50	5.15	12.65		

in the presence of benzaldehyde is insoluble in acids, while in the hydrolysate of fibrin alone, approximately 45 per cent of the humin nitrogen was acid-soluble, forming a jet-black solution in the diluted hydrochloric acid, but being completely adsorbed by or combining with the lime in the ammonia determination. Just what significance lies in the "acid-insoluble" and "acid-soluble" figures will be made a subject for further investigation.

Hydrolysis of Fibrin in the Presence of Formaldehyde.—The experiments with furfural and benzaldehyde were repeated, using instead 0.5, 1.0, 2.0, and 4.0 cc. of a 40 per cent solution of formaldehyde. The figures obtained for those experiments where 0.5 and 4.0 cc. were used, were so unexpected that they were repeated at a later date and, to my surprise, were confirmed. The data obtained are shown in Table XII.

It will be seen that here, again, we are dealing with a number of chemical reactions. The ammonia nitrogen falls significantly when small quantities of formaldehyde are added, but rises very rapidly when larger quantities are present. This rise must be due to deamination, but with the data at hand it is impossible to obtain any definite idea of the reactions involved.

In all cases the filtrate from the acid-insoluble humin was dark red-brown, in decided contrast to that from the experiments where furfural or benzaldehyde was used. Only small quantities of

TABLE XII.

The Effect on Ammonia N and Humin N of Hydrolyzing 8 Gm. of Fibrin in the Presence of Varying Amounts of Formaldehyde.

40 per cent formaldehyde added.	Ammonia N.	Change (average).	Acid-insoluble humin.	Acid-soluble humin.	Total humin.	Increase (average).	
cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
None (average).	46.42*				13.13*		
None (average).†	45.20		7.00	5.35	12.35		
0.5	40.45		9.75	22.15	31.90		
		− 5.92				19.02	144.8
0.5	40.55		8.35	23.85	32.20		
1.0	42.35	− 4.07	2.15	31.80	33.95	20.82	158.6
2.0	50.55	+ 4.13	1.60	34.25	35.85	22.72	173.0
4.0	69.55		1.20	30.15	31.35		
		+21.56				18.75	142.8
4.0	66.40		2.05	30.35	32.40		

* Used as a standard check in all the tables.

† Data of Table XI.

acid-insoluble humin were obtained from those experiments where 1 cc. or more of formaldehyde was present, and this humin lacked the intense black color normally present, being decidedly brown. The acid filtrate, on concentration, yielded a red-brown resin, which was easily soluble in hot water, forming a red-brown solution. The filtrate from the acid-soluble humin, in the experiment where 4 cc. of formaldehyde were used, was *dark red* but clear. The red color diminished in intensity down to 0.5 cc. of formaldehyde, where the color was but slightly deeper yellow than normal.

With the data at hand it is impossible to determine what reactions are involved in the initial gain of approximately 30 per cent in acid-insoluble nitrogen in the case where 0.5 cc. of HCHO was added, followed almost at once by a rapid fall, amounting to a loss of nearly 70 per cent of the insoluble humin when 1.0 cc. of HCHO was added. The insoluble humin N which was lost appears to be recovered in the "acid-soluble" humin N but it is impossible to state whether this is actually the case or whether it remains in the filtrate from the humin.

The Nitrogen Distribution of Tyrosine and Tryptophane when

Boiled with Benzaldehyde and Formaldehyde.—In view of the peculiar results obtained by hydrolyzing fibrin in the presence of benzaldehyde and formaldehyde, it was thought advisable to test out the effect of these aldehydes on individual amino-acids. The only amino-acids which were available at this time were tryptophane and tyrosine. Both of these compounds are of special interest. Tryptophane is important in view of my belief (Gortner and Blish, 1915) that a very considerable portion of the humin nitrogen of *protein* hydrolysis has its origin in the tryptophane molecule.

Tyrosine should be of special importance in a study involving formaldehyde and hydrochloric acid. It has long been known that phenols will react with formaldehyde to form various products depending upon the conditions under which the reaction is carried out[5] and it seemed very probable that tyrosine, because of its hydroxyl group, would form a resin with formaldehyde when heated in the presence of strong hydrochloric acid.

When formaldehyde was added to tryptophane + HCl, a light yellow-gray precipitate separated in the cold. This is probably Miss Homer's (1913, a) crystalline condensation product, although the granules showed no crystal form under a magnification of 600 diameters. On warming, the solution rapidly became black and a very considerable amount of black insoluble humin separated. The acid filtrate from the insoluble humin was in every instance deep red. This red color remained in the "soluble humin" fraction, the filtrate from the soluble humin having the usual yellowish color.

Adding benzaldehyde to the tryptophane + HCl and warming produced an intense black coloration. Within a short time after boiling began, however, the insoluble humin "balled" together, leaving the liquid light yellow. The small amount of "soluble humin" was snow white, and was probably only lime.

Tyrosine + benzaldehyde + HCl, on heating, did not develop a black color throughout the solution, the only blackening which occurred seemed to be in the benzaldehyde floating upon the surface of the acid. This gradually darkened so as to be almost

[5] For the literature and a discussion of these reactions, see Baekeland (1909, a and b).

TABLE XIII.

The Nitrogen Distribution of Tyrosine and Tryptophane when Boiled for 48 Hours with Benzaldehyde or Formaldehyde in the Presence of Hydrochloric Acid.

Aldehyde added.		Amino-acid added.		Ammonia N found.	"Insoluble humin" N found.	"Acid-soluble" humin N found.	Total humin N found.	Per cent of total N in total humin.	N in filtrate from humin.	Per cent of total N recovered
cc.			*gm.*	*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	
None.		Tyrosine +	0.2028							
		tryptophane.	0.1134	0.45	None.	1.35				
Benzaldehyde, 2		Tryptophane.	0.1200	2.85			10.15	61.66	Lost.	
"	3	"	0.1220	3.10	12.35	0.05	12.40	74.11	1.05	98.93
"	3	Tyrosine.	0.2020	0.25	11.95	2.35	14.30	91.43	1.55	102.9
40 per cent formaldehyde, 2		"	0.2000	0.35	None.	10.25	10.25	65.97	5.15*	101.4
40 " " "	2	"	0.3010	0.55	"	18.10	18.10	77.67	3.70	95.92
40 " " "	5	"	0.3010	1.55	"	8.10	8.10	34.76	13.40	98.91
40 " " "	5	"	0.2802	1.55	"	9.04†	9.04†	41.68	11.10	100‡
40 " " "	2	Tryptophane.	0.1246	1.85	9.55	2.00	11.55	67.54	3.50	98.83
40 " " "	5	"	0.1654	2.50	9.70	3.15	12.85	56.61	6.35	95.61

* Humin washed with boiling water. Probably some of the resin is soluble in hot water for flocks of solid separated in the filtrate. The remaining determinations were washed with cold water.

† By difference from total N. Actual determination lost in Kjeldahling.

‡ Calculated.

black, and on cooling, solidified to a black mass. The filtrate from the acid-insoluble humin was bright orange.

On boiling tyrosine with formaldehyde + HCl, no black coloration appeared. The solution darkened slightly, however, a red-yellow coloration being produced. On evaporating off the HCl, a red-yellow resin was left, which easily dissolved in hot water. The calcium precipitate (soluble humin) was light yellow, gelatinous, and filtered slowly. The filtrate from the soluble humin was bright orange, the color deepening with increasing amounts of formaldehyde.

The experimental data are given in Table XIII. It is useless to discuss the individual experiments until additional data are obtained. Several notable differences are, however, apparent.

1. The ammonia determinations show that deamination of both amino-acids takes place under the influence of either aldehyde, and that this deamination occurs somewhat more readily in the case of tryptophane.

2. Heating with HCl in the presence of benzaldehyde causes a large percentage of the nitrogen of both tryptophane and tyrosine to remain in the "acid-insoluble" humin.

3. Heating with HCl in the presence of formaldehyde produces a very considerable amount of acid-insoluble humin in the case of tryptophane and no acid-insoluble humin in the case of tyrosine. A very considerable proportion of the tyrosine nitrogen is retained in the acid-soluble humin.

4. Increasing the amount of formaldehyde used *decreases* the yield of "humin" nitrogen formed from tyrosine, the nitrogen returning to a form in which it is not precipitated by calcium hydroxide.

SUMMARY.

1. The figures for ammonia N in a protein hydrolysate are not significantly altered when the hydrolysis is carried out in the presence of a quantity of carbohydrate equal to three times the weight of the protein material.

2. Humin nitrogen is greatly increased by the addition of carbohydrate material. This increase is probably due both to chemical and physical causes.

3. Tryptophane cannot be accurately estimated by hydrolyzing proteins in the presence of carbohydrates.

4. If the weight of carbohydrate material present during protein hydrolysis greatly exceeds the amount of protein, an accurate nitrogen distribution, by Van Slyke's method, cannot be obtained.

5. Attention is called to the fact that, when nitrogenous compounds other than proteins are present in a hydrolysate, no reliance can be placed upon the figures obtained in any of the fractions, as representing actual amino-acids. Such data should not be compared with those obtained by the analysis of pure proteins.

6. When fibrin is hydrolyzed in the presence of furfural, the humin nitrogen is greatly increased, but is seems probable that this increase is due, not only to a chemical reaction, in which certain amino-acids combine with furfural, but also to adsorption or occlusion of other amino-acid nitrogen by the humin.

7. When furfural is boiled with strong hydrochloric acid approximately 75 per cent by weight is converted into a black insoluble mass. It is suggested that perhaps the humin, formed from carbohydrates by boiling with hydrochloric acid, is actually formed from furfural, which is in turn formed from the carbohydrate.

8. When fibrin is hydrolyzed in the presence of benzaldehyde, the humin nitrogen rises rapidly to a maximum of approximately double the amount produced by ordinary hydrolysis. This humin nitrogen is nearly all "acid-insoluble." The reaction here appears to be wholly chemical. The ammonia nitrogen is not significantly altered, although there is evidence that some deamination takes place.

9. When fibrin is hydrolyzed in the presence of formaldehyde, there is an initial gain of "acid-insoluble" humin where small amounts of formaldehyde are used, but a large loss when greater quantitites are present. The total humin nitrogen (acid-insoluble + acid-soluble humin) is fairly uniform for all quantities of formaldehyde, and shows an increase of approximately 150 per cent of the normal humin nitrogen. Deamination occurs in the presence of the larger quantities of formaldehyde.

10. Both tryptophane and tyrosine yield a very considerable

proportion of "acid-insoluble" humin nitrogen, when boiled with hydrochloric acid in the presence of benzaldehyde.

11. When tryptophane is boiled with formaldehyde, in the presence of hydrochloric acid, a very considerable part of the nitrogen is retained in the acid-insoluble humin. This is in decided contrast to the behavior of tyrosine, where no acid-insoluble humin is formed, but where a greater or less percentage of the nitrogen is retained in the "acid-soluble" humin, the amount retained depending upon the quantity of formaldehyde present. Some deamination occurs in both amino-acids, when heated with either aldehyde, in the presence of hydrochloric acid.

The work is being continued along lines suggested by these results.

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THE URIC ACID SOLVENT POWER OF NORMAL URINE.

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Very little work has been done to determine the amount of uric acid which urines from normal individuals can take up by being shaken with pure uric acid. The only investigations of consequence are those of Hindhede[1] and Blatherwick.[2]

Certain researches reported elsewhere by the author[3] involved the determination of the power of normal urines to dissolve uric acid, and it may be of interest to report the findings in full in this paper. We had hoped to discover all the main factors on which the uric acid dissolving power of urines depends, so as to be able on the basis of certain data to foretell approximately how much uric acid would be dissolved by a particular urine. In this we were doomed to disappointment. The only factors which we have proved to play a definite part are the acidity of the urine and its concentration. Some urines that are slightly acid and all those that are neutral or alkaline dissolve extra uric acid. On the other hand the solvent power of urines of ordinary concentration is distinctly increased by dilution. For instance, the total uric acid dissolved by 100 cc. of urine after being diluted with 300 cc. of distilled water may be even twice as much as that dissolved by 100 cc. of undiluted urine. This will explain why we have reported the dilute urines in a separate table.

[1] Hindhede, M., *Skand. Arch. Physiol.*, 1912, xxvi, 384.

[2] Blatherwick, N. R., *Arch. Int. Med.*, 1914, xiv, 409.

[3] Haskins, H. D., *Arch. Int. Med.*, 1915, xvi, 1055; 1916, xvii, 405.

TABLE I.

Solvent Action of Dilute Urines.

Urine No.	Acidity, P_H.	Uric acid content.	Total uric acid.	Uric acid dissolved.
		Mg. per 100 cc.		
1	7.3	9.8	154.5	144.8
2	7.1	15.0	172.3	157.3
3	7.0	20.4	138.8	118.4
4	6.95	16.5	122.7	105.7
5	6.95	8.4	78.0	69.6
6	6.95	9.2	67.6	58.4
7	6.9	8.3	150.1	141.8
8	6.9	9.0	139.1	130.1
9	6.9	8.3	120.0	111.8
10	6.85	8.3	144.5	136.2
11	6.85	19.4	128.7	109.3
12	6.8	18.8	135.6	116.8
13	6.8	13.2	121.2	108.0
14	6.8	7.5	109.7	102.2
15	6.8	8.2	90.4	82.1
16	6.8	11.1	89.3	78.2
17	6.8	9.2	70.0	60.8
18	6.75	8.3	91.3	83.0
19	6.75	12.0	89.3	77.3
20	6.75	9.0	75.6	66.6
21	6.75	8.8	66.0	57.2
22	6.7	8.3	105.0	96.8
23	6.7	19.6	86.0	66.4
24	6.7	15.5	81.9	66.4
25	6.7	7.5	69.0	61.5
26	6.7	28.5	87.5	59.0
27	6.7	8.4	56.4	48.0
28	6.7	15.2	48.6	33.4
29	6.65	18.0	252.4	234.4
30	6.65	7.5	73.5	66.0
31	6.65	15.2	69.6	54.4
32	6.65	7.5	51.1	43.7
33	6.6	7.5	83.3	75.8
34	6.6	18.7	93.1	74.4
35	6.6	9.0	72.0	63.0
36	6.6	7.1	61.5	54.4
37	6.6	9.0	59.3	50.3
38	6.6	9.2	50.0	40.8
39	6.55	15.0	62.9	48.0
40	6.55	20.6	57.8	37.3

TABLE I—*Concluded.*

Urine No.	Acidity, P_H.	Uric acid content.	Total uric acid.	Uric acid dissolved.
		Mg. per 100 cc.		
41	6.5	12.2	76.2	63.9
42	6.5	15.2	66.0	50.8
43	6.5	9.7	59.3	49.6
44	6.5	8.2	50.4	42.2
45	6.5	22.0	54.0	32.0
46	6.45	10.8	72.4	61.6
47	6.45	7.6	59.0	51.5
48	6.45	7.6	41.2	33.6
49	6.45	22.8	55.6	32.8
50	6.45	8.4	26.8	18.4
51	6.4	19.7	68.4	48.8
52	6.4	28.0	74.9	47.0
53	6.4	7.6	38.8	31.2
54	6.4	24.8	52.4	27.6
55	6.35	7.5	30.4	22.9
56	6.35	7.6	26.8	19.2
57	6.35	8.4	14.0	5.6
58	6.3	9.0	106.5	97.5
59	6.3	7.5	41.3	33.8
60	6.3	12.4	31.6	19.2
61	6.25	7.6	27.1	19.5
62	6.25	7.6	21.2	13.6
63	6.2	15.6	22.8	7.2
64	6.0	14.4	52.3	37.9
65	6.0	10.0	22.0	12.0
66	5.95	10.7	25.5	14.8
67	5.8	9.2	29.2	20.0

TABLE II.

Solvent Action of Less Dilute Urines.

Urine No.	Acidity, P_H.	Uric acid content.	Total uric acid.	Uric acid dissolved.
		Mg. per 100 cc.		
68	7.2	45.8	234.3	188.5
69	7.2	61.9	250.1	188.2
70	7.2	56.3	238.4	182.0
71	7.1	72.4	244.4	172.0
72	7.1	50.4	185.8	135.4
73	7.1	91.8	210.2	118.4
74	7.1	96.8	202.5	105.8
75	7.0	63.8	291.8	228.0
76	7.0	70.1	276.0	205.9
77	7.0	49.2	247.6	198.4
78	7.0	52.4	240.0	187.6
79	7.0	54.8	234.8	180.0
80	7.0	45.2	207.6	162.4
81	7.0	46.8	183.6	136.8
82	7.0	87.6	193.2	105.6
83	6.95	44.4	142.8	98.4
84	6.95	27.6	122.0	94.4
85	6.95	70.8	162.8	92.0
86	6.95	36.4	120.4	84.0
87	6.95	47.4	123.7	76.2
88	6.95	40.4	105.2	64.8
89	6.95	82.0	144.4	62.4
90	6.95	55.6	114.4	58.8
91	6.9	37.5	343.1	305.7
92	6.9	122.8	282.0	159.2
93	6.9	71.4	230.2	158.8
94	6.9	34.5	137.3	102.8
95	6.8	62.0	216.4	154.4
96	6.8	42.8	174.0	131.2
97	6.8	31.6	145.2	113.6
98	6.8	42.8	145.2	102.4
99	6.8	87.6	165.2	77.6
100	6.8	41.2	114.0	72.8
101	6.8	51.6	122.0	70.4
102	6.8	47.6	101.2	53.6
103	6.8	52.4	102.0	49.6
104	6.8	60.8	100.0	39.2
105	6.8	38.8	74.0	35.2
106	6.7	50.0	198.0	148.0
107	6.7	78.8	180.4	101.6

TABLE II—*Concluded.*

Urine No.	Acidity, P_H.	Uric acid content.	Total uric acid.	Uric acid dissolved.
		Mg. per 100 cc.		
108	6.7	58.0	134.0	76.0
109	6.7	27.1	95.7	68.6
110	6.7	46.0	107.6	61.6
111	6.7	53.2	102.0	48.8
112	6.7	56.0	104.7	48.7
113	6.65	50.0	186.8	136.8
114	6.65	26.8	93.2	66.4
115	6.65	74.0	105.2	31.2
116	6.65	29.2	60.4	31.2
117	6.6	26.8	123.6	96.8
118	6.6	43.1	136.3	93.2
119	6.6	83.0	158.5	75.5
120	6.5	42.0	73.0	31.0
121	6.5	42.0	68.4	26.4
122	6.5	66.0	78.8	12.8
123	6.45	41.2	100.4	59.2
124	6.45	59.6	88.4	28.8
125	6.45	34.0	62.0	28.0
126	6.45	40.8	67.4	26.6
127	6.45	38.0	62.0	24.0
128	6.45	56.4	65.2	8.8
129	6.4	44.4	75.6	31.2
130	6.4	26.8	52.4	25.6
131	6.4	29.2	54.0	24.8
132	6.4	57.2	66.8	9.6
133	6.35	78.6	108.0	29.4
134	6.3	57.2	72.4	15.2
135	6.3	28.4	42.0	13.6
136	6.3	68.4	79.6	11.2
137	6.3	55.6	66.0	10.4
138	6.25	95.5	102.9	7.4
139	6.1	25.2	34.0	8.8
140	5.9	35.6	76.2	40.6
141	5.8	25.8	44.8	19.0

Methods.

In determining solvent power the urines were first warmed to 37°C., and after adding an excess of pure uric acid the flasks were kept in a bath at 37°C. for exactly 20 minutes, the urines being shaken frequently so that undissolved uric acid was constantly in suspension. They were filtered immediately after removal from the bath, and the uric acid estimation was made on the filtrates. A longer period of warming was not found to give superior results. Body temperature was chosen so as to be comparable to the physiological condition of urine lying in the bladder.

The uric acid estimations were made by the usual method; *i.e.*, by precipitation by means of ammonium hydroxide in the presence of a considerable concentration of ammonium sulfate, filtration, and subsequent solution of the precipitate in hot dilute sulfuric acid and its estimation by titration with potassium permanganate.

The hydrogen ion concentration or acidity of the urines was determined by the method of Henderson and Palmer.[4]

The figures in the tables for acidity of the urines are the "hydrogen exponents" (P_H); these are (negative) logarithmic figures corresponding to the true hydrogen ion concentrations. The smaller the figure the greater the acidity. Neutrality is 7.0. The reaction of normal urines which we have examined ranges from a distinctly acid urine, 5.0, to a slightly alkaline urine, 7.4.

Hindhede's method of determining solvent power of urines was found to be quite unsatisfactory. Our work was carried out independently, being uninfluenced by the investigations of others. It is necessary to say a word in regard to Blatherwick's method. It is quite possible that by his method of shaking with uric acid for 2 hours at room temperature somewhat different results would be obtained than by our method of shaking for 20 minutes at body temperature. We would, therefore, warn against comparing the results by the two methods.

Tables I and II give the results of the experimental work. The figures for uric acid are given as mg. in 100 cc. of urine. "Uric acid content" means the amount present in the urine as passed. "Total uric acid" means the amount present in the filtrate after shaking with uric acid. The difference between these two is the "uric acid dissolved." This last is the index of the solvent power of the urine.

From a study of the tables it will be seen that many acid urines (5.8 to 6.9 acidity) have the power to take up additional uric acid, the amount being greater than distilled water will dissolve; namely, 8.4 mg. per 100 cc. The uric acid content of many of these urines (see Table II) is as great as occurs during an ordinary high excretion of uric acid (50 to 70 mg.). Many urines

[4] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912-13, xiii, 393.

of an acidity between 5.8 and 6.3, which were tested, dissolved no extra uric acid and therefore were not included in the tables. It was most surprising to find solvent power in *any* urines of that range of acidity. The result with the urine of 5.9 acidity (No. 140) was quite remarkable. In the case of urines of acidity 6.4 to 7.4 almost every one that we have tested showed uric acid solvent power.

It will be observed that in a general way the amount of uric acid dissolved increases as the acidity of the urine decreases (*i.e.*, as the acidity figure becomes greater). Disregarding a few of the exceptionally high estimations and a number of the exceptionally low ones, the extremes of the amounts of uric acid dissolved for various ranges of acidity are about as follows:

Acidity.	Uric acid dissolved.
5.8 to 6.35	5 to 40 mg. (both dilute and less dilute urines).
6.4 " 6.55	25 " 60 " " " " " " "
6.6 " 6.7	40 " 100 " " " " " " " "
6.75 " 6.8	50 " 115 " (dilute); up to 155 mg. (less dilute).
6.85 " 6.9	105 " 140 " " " " 160 " " "
6.95 " 7.0	105 " 120 " " " " 225 " " "
7.1 " 7.3	145 " 155 " " 105 to 190 " " "

It does not seem to us advisable to calculate averages for the amount dissolved by urines of the same acidity, as it would be misleading because of the wide variations in the amounts. For example, for acidity 6.8 (Table II) the figures run from 35 to 154 mg.

A number of the urines show exceptionally high solvent power as compared with others of the same acidity, notably 29 and 58 in Table I, also 91, 106, 113, and 140 in Table II.

In order to determine whether the inorganic constituents of the urine could of themselves account for the solvent power of any particular urine, 700 cc. of urine were evaporated and ashed, then the ash was redissolved in 700 cc. of distilled water. On shaking with uric acid the ash solution dissolved 39 mg. per 100 cc. while the original urine took up 40 mg. In the case of this urine at least it was probable that the uric acid was dissolved because of a reaction with disodium phosphate producing mono-

sodium phosphate and monosodium urate. This reaction occurs readily with mixtures containing only the two phosphates, these mixtures resembling urines in so far as concerns acidity and phosphate content. Urines of different acidities contain different proportions of Na_2HPO_4 and NaH_2PO_4; *e.g.*, a urine of acidity 6.8 contains equimolecular quantities of the two, but one of acidity 7.4 contains about four times as much Na_2HPO_4 as NaH_2PO_4.

Solutions having different concentrations of the phosphates but having the same acidity show a power to dissolve uric acid in a ratio entirely different from that of the dilution. This has a bearing on the fact which is made apparent by a comparison of the results reported in Tables I and II; namely, that the dilute urines have a solvent power out of proportion to their concentration. The results of the experiment with phosphate mixtures are given in Table III.

TABLE III.

Solvent Action of Phosphate Mixtures.

No.	Acidity, P_H.	Uric acid dissolved, mg. per 100 cc.	Gram molecular concentration.
1	6.8	138.2	1/15
2	6.8	120.0	1/30
3	6.8	75.5	1/60
4	6.9	90.0	1/50
5	6.9	58.8	1/100
6	6.8	78.8	1/50
7	6.8	50.0	1/100
8	6.65	54.4	1/50
9	6.65	39.6	1/100

It will be seen that the solvent power is not nearly proportional to the concentration, for instance, Solution 3 is only one-fourth as concentrated as Solution 1 but dissolves more than half as much uric acid as 1. Compare also 5 with 4, 7 with 6, and 9 with 8. Similar results were obtained with urines after dilution with one and with three volumes of water (Table IV).

TABLE IV.

Solvent Action of Diluted Urines.

Dilution.	Acidity, P_H.	Uric acid content.	Total uric acid.	Uric acid dissolved.
		Mg. per 100 cc.		
Urine A.....	6.8	70.0	162.8	92.8
50 per cent of A.....	6.8	35.0	112.4	77.4
25 " " " "	6.75	17.5	80.4	62.9
Urine B.....	6.4	10.8	86.0	75.2
50 per cent of B.....	6.35	5.4	53.2	47.8
25 " " " "	6.3	2.7	30.4	27.7

The solvent power of certain very dilute urines was one of the most surprising results of our investigation. One sample of urine passed as a consequence of excessive drinking of water, having a specific gravity of 1.0005 (6.6 acidity), dissolved 54.4 mg. of uric acid per 100 cc. In the case of two other persons who drank large quantities of water the urine was of similar character. One passed 4,800 cc. in 24 hours (acidity 6.75), and 100 cc. of this dissolved 56 mg.; while the other passed 2,800 cc. (acidity 6.8), 100 cc. dissolving 114 mg. It is possible that one of the important factors involved in dilution is decrease in concentration of sodium ions. By what is called the "common ion effect" the solubility of a salt is lessened whenever another salt is present, one of whose ions is identical with an ion of the salt in question. The presence of sodium ions (*e.g.*, from NaCl) in the urine must therefore depress the solubility of monosodium urate, and the more dilute the urine the less the depression of solubility because of the smaller number of ions present. In the case of simple aqueous solutions the sodium ion effect is readily demonstrated, Monosodium urate dissolves in water so that a solution is easily obtained which contains an amount of urate equivalent to 170 mg. of uric acid in 100 cc. On the other hand solutions of four different sodium salts (each containing the same amount of sodium as a 0.3 per cent NaCl solution) dissolved only a trace of sodium urate. In solutions of potassium salts the urate dissolved quite readily.

For some reason this ion effect does not seem to be so marked in the case of urine and phosphate mixtures. The addition of

1 per cent NaCl to a phosphate mixture (acidity 6.8) diminished the uric acid dissolved from 120 mg. to 105. Also in the case of a urine which showed great solvent power (206 mg. per 100 cc.) the addition of NaCl had little effect (200 mg. dissolved). These findings seem to minimize somewhat the importance of the ion effect.

By referring to Tables I and II it will be seen that a considerable number of urines (twenty-four) show figures for "total uric acid" greater than the uric acid content of a saturated solution of sodium urate (170 mg.). Figures for total uric acid of 252, 276, 282, 291, and 343 can hardly be explained on the basis of solution occurring as the result of a reaction producing monosodium urate. There must be some other factor responsible for the apparent supersaturation. Urines passed after taking sodium bicarbonate or citrate behave in a similar manner (Table V).

TABLE V.

Solvent Action of Urine after Sodium Citrate and Bicarbonate.

Acidity, P_H.	Salt taken.	Uric acid content.	Total uric acid.	Uric acid dissolved.
		Mg. per 100 cc.		
7.6	Bicarbonate.	13.8	296.9	283.1
7.3	"	96.6	446.6	350.0
7.3	"	105.0	360.8	255.8
7.3	Citrate.	70.5	283.9	213.4
7.3	"	129.2	313.9	184.6
7.25	"	56.0	227.2	171.2
7.25	"	72.0	222.3	150.3
7.2	Bicarbonate.	55.5	279.0	223.5
7.2	Citrate.	52.0	218.9	166.9
7.15	"	27.0	210.4	183.4
7.1	"	30.6	197.9	167.3

The most probable explanation of this apparent supersaturation is that at least part of the uric acid is in colloidal solution. Schade and Boden[5] have shown that dilute alkalies can cause colloidal solution of uric acid at body temperature. By careful manipulation they put 500 mg. of uric acid into solution in

[5] Schade, H., and Boden, E., *Z. physiol. Chem.*, 1913, lxxxiii, 347.

40 cc. of dilute sodium hydroxide. They believe that the colloids of the blood stabilize colloidal uric acid, and also that colloids in the urine can exert the same action so that uric acid can exist in it in colloidal form. It is not necessarily true that *all* of the uric acid taken up by any of our urines was put into colloidal solution, part of that dissolved may have become monosodium urate. We noticed that the precipitate from some urines after adding ammonium hydroxide was gelatinous, quite unlike the usual ammonium urate precipitate; these may have held uric acid in colloidal solution. No record was made of the particular urines that behaved in this manner.

Bechhold[6] has confirmed the existence of colloidal solutions of uric acid by the use of the ultra filter, the solution remaining on the filter becoming very much more concentrated in uric acid because the filter holds back the colloidal particles.

SUMMARY.

1. When shaken with uric acid for 20 minutes at 37°C. many urines that are slightly acid and all that are neutral or alkaline take up extra uric acid.

2. The less acid the urine the more uric acid, as a rule, it will dissolve.

3. Dilute urines when considered in proportion to their concentration show much greater solvent power than less dilute urines.

4. Some urines dissolve so much uric acid that they come to contain more uric acid than is present in a saturated solution of monosodium urate. In all probability in these cases at least part of the uric acid is in colloidal solution.

[6] Bechhold, H., *Z. Electrochem.*, 1914, xx, 321.

CELL PENETRATION BY ACIDS.

II. FURTHER OBSERVATIONS ON THE BLUE PIGMENT OF CHROMODORIS ZEBRA.

By W. J. CROZIER.

Contributions from the Bermuda Biological Station for Research, No. 48.

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I. The blue substance occurring in the tissues of the large nudibranch *Chromodoris zebra* Heilprin has been employed as an intracellular indicator for acids, which on penetrating the tissues cause the material to become pink (Crozier, 1916). This paper records some further observations with reference to the action of the pigment as an indicator and to its participation in the natural color variations of *Chromodoris*.

It was at first considered not impossible that more than one substance might be concerned in the color change produced by acids. Smallwood (1910, p. 139) states that the five or six *white* conical elevations on the posterior border of the mantle of *Chromodoris* turn pink when treated with nitric acid; this statement, however, certainly involves an error, because the action of acids does not in any way affect the color of the creamy white contents of these glandular organs. Smallwood (unaware of the nature of the blue substance) was probably misled by the diffusion of pigment from neighboring portions of the integument, or by the pink hue assumed in pigment-bearing cells external to the body of these white glands.

It can be stated quite definitely that the blue substance is the only indicator involved, but its reactions are sufficiently complicated to require some attention. It would be of interest, in this connection, to examine the apparently similar blue pigments of other species of *Chromodoris*. According to Eliot's description (1913, p. 29, pl. II, fig. 9) the Japanese *Chromodoris marenzelleri* Bergh is colored in such a way as to suggest a skin pigment allied to that of *C. zebra*, as is also the case with some Pacific coast species (Cockerell, 1901).

II. The blue pigment also occurs dissolved in the blood of *Chromodoris*. The blood pigment in its reactions to alkalies and acids

is identical with that obtained from the cells of the foot and general integument. The absorption spectra are likewise the same, a single band being present, which in neutral solutions centers at about 621 $\mu\mu$ (Crozier, 1914). Blood obtained by means of a syringe in such a way as to be kept from contact with air is of a deep blue, almost violet, color by reflected light, reddish purple by transmitted light. The reaction of blood obtained from the auricle was estimated from a number of analyses to be about $p_H = 7.4$ (20°C.), the procedure of Rowntree, Marriott, and Levy (1915) being used for this purpose.

Qualitative tests indicate the presence of manganese in the blood and in partially purified pigment extracts, but I am unable to say whether or not manganese forms a constituent of the pigment substance.

III. Undiluted blood and aqueous solutions of the pigment obtained from the integument were allowed to stand (a) in open vessels and (b) in tightly stoppered bottles; some of the latter contained air while others were completely filled with fluid. At 27°C. the pigment not in contact with air was rapidly decolorized; it was determined that the presence of pieces of tissue accelerated the decolorization. The reduction was slower at 20° than at 27°. In bottles holding air above the pigment solution the upper layer remained blue for some time, but ultimately was completely decolorized; in this case the blue color could be regenerated several times by shaking. The admission of fresh air caused these solutions again to become blue, provided they had not stood too long. The pigment solutions contained in open vessels were also decolorized after the formation of a bacterial film.

The addition of neutral H_2O_2 immediately produced a permanent intense blue coloration, or a violet tint showing blue fluorescence. This color was much more intense than could be produced by long shaking with air.

During the progress of decolorization there was never any indication of a pink stage. The color change with acids is therefore not a reduction.

Extracts of the pigment made with boiling water and subsequently preserved in closed vessels were not reduced, nor was there any evidence of reduction when the extraction was made with boiling water in a flask stoppered with cotton wool. Ex-

tracts prepared in the cold were not reduced in the presence of formaldehyde, $CHCl_3$, xylol, acetone, or pyridine. Numerous experiments were made by adding to solutions of the blue pigment, sterilized with $CHCl_3$, portions of the body of *Chromodoris* either whole or ground with sand. No reduction was obtained in this way even when the tests were carried out in tightly closed bottles containing no free air. Negative results were likewise obtained with the liver of fishes, which yields, according to Harris and Creighton (1915), a non-specific reductase rapidly affecting hemoglobin.

The reduction of this *Chromodoris* pigment seems, therefore, to be strictly comparable to that (oxyhemocyanin) found by Alsberg (1915) in the case of *Limulus*. The reduction is not due to enzymes, but to the action of putrefactive microorganisms. If to an aqueous extract of the pigment that has been sterilized by boiling there be added a similar solution that has become reduced, the inoculated flask is likewise slowly decolorized. When allowed to stand too long, the blue color cannot be restored even with H_2O_2, indicating that the blue substance has been destroyed.

Reduction of the blue material by direct chemical means is not easily effected. It is not decolorized by boiling, either alone or with acid, and it is only slowly reduced with zinc dust and acid. In the neutral or slightly alkaline condition the blue pigment is partially reduced to a greenish tint by Stoke's reagent, but it is not completely decolorized. Acid solutions are not reduced by Stoke's reagent. These tests with freshly prepared Stoke's reagent were checked spectroscopically.

Whether or not the blue pigment is concerned in respiration cannot as yet be decided, but its behavior certainly suggests that it may serve as an oxygen carrier. There is reason to believe that the metabolism of sluggish marine invertebrates, such as *Chromodoris*, is not of a rapid type (Vernon, 1895) and the difficulty with which the pigment seems to be reduced may thus be no real objection to its fulfilling a respiratory function. Some experiments made to test this question have not given definite results.

The method used in my previous work of handling the pieces of tissue has one advantage not mentioned in my first paper. In pressing the tissue gently between filter paper before submitting it to the action of acid, any blood pigment held in lacunar peripheral blood spaces is likely to be removed. Fortunately, it is

possible to make a decisive test showing the absence of errors from this source. Some *Chromodoris* individuals show a pure white at the edge of the mantle, although their blood is blue, as is also the surface of the foot and other portions of the skin. Pieces of this white skin tissue do not turn pink with acid, even when first treated with H_2O_2 to oxidize any blood pigment present.

IV. Several of the factors involved in determining variations in the blue coloration of this animal may be distinguished: the presence or absence of the pigment in the integument, the details of its local distribution, the extent to which it is oxidized, the proportion of the pigment which is firmly bound by the cell protoplasm as compared with that present in solution, and the reaction of the fluid locally associated with the dissolved form of the pigment. The last two factors enumerated are the most important from the standpoint of the use of the blue pigment as an intracellular indicator. It should also be stated that the oxidized condition of the pigment is the only one which is turned pink by acids; if acid is added to a reduced solution, the solution remains colorless, becoming pink only when shaken with air or treated with H_2O_2.

These complicating factors are important in determining variations found in the acid penetration of mantle tissue from variously pigmented individuals; they are probably as much concerned at least as is the variable judgment of the observer, which I at first believed mainly responsible. As just stated, the cells of the blue integument appear to contain the pigment in two conditions: (a) a relatively free (dissolved) state, and (b) bound by the cell protoplasm in a firm combination which, as previously noted in the case of the dorsal skin, is affected only by strong acids. When pieces of the mantle are immersed in weak acid solutions the pink hue may disappear after a time, leaving the tissue blue; this is due to the fact that only the dissolved (acid-sensitive) portion of the pigment is diffusible. In working at low temperatures (15°) a serious source of confusion may arise here, since the outward diffusion of pigment is frequently more rapid than the inward progress of the acid. This difficulty is, however, practically absent in freshly collected animals. With the object of following the changes in pigmentation I have studied animals maintained in the laboratory for 3 to 4 months without food, and I

am convinced that in these individuals the conditions as regards the blue pigment are materially altered from those normally obtaining. There is an increase in the relative amount of pigment not lost by diffusion into distilled water or acid, and the diffusible portion of the pigment is more *quickly* lost to a surrounding solution. The penetration time of all acids tested is much less than in freshly collected animals.

In discussing the results obtained by observing the comparative penetration speed of acids (Crozier, 1916), two circumstances were not dealt with which may be mentioned here. One is the possibility that the tissue does not remain inert when exposed to the action of acid; but is (in a sense) stimulated to oppose the entrance of the foreign material; the nature of this possible effect may be such as to involve the partial coagulation of the cell substance and an alteration in the relations of the internal pigment. Closely allied to this is the second possibility; namely, that coagulation may be induced by a variable fraction of the acid, while the remainder can affect the indicator less readily when the latter is bound (?) by coagulated protein. This source of objection is removed, I believe, by the following experiment:

When pieces of tissue which have been turned pink by acid are transferred to sea water, the acid diffuses away and sea water enters. This occurs within a few minutes, usually, depending on the acid and its concentration. The pink hue is then lost, the tissue becoming greenish blue because of the sea water alkalinity. If these pieces of tissue be then placed again in acid, they become pink almost instantly. This is true even with weak acids, as 0.05 N acetic.

V. The purple state of the indicator is the one most sensitive to the addition of acids, or rather, the solutions containing it are intermediate in actual acidity between those producing the blue and those producing the pink states. The small pigment globules within the cell may in some cases be of higher acidity than obtains in other cases, or in the rest of the cell contents; as Osterhout (1913) clearly pointed out, there are many semipermeable surfaces, probably of different qualities, comprised in the structure of a single cell. This means that, even if the protoplasm of blue and purple mantle tissues were penetrated by acids with equal ease, still in measuring penetration with this indicator the lowest

figures should be obtained with the purple tissue. This is actually the case, as was noted previously (Crozier, 1916, p. 261), and has since been confirmed by the use of a number of acids not employed in the paper referred to.

SUMMARY.

VI. 1. The blue integumentary pigment of *Chromodoris zebra* Heilprin is found also in the blood of this nudibranch. It seems resistant toward reducing agents, but is reduced to a colorless condition by the action of putrefactive microorganisms. In this respect the *Chromodoris* pigment resembles oxyhemocyanin. The blue color is regenerated on shaking with air or adding H_2O_2. Only in the oxidized condition is it turned pink by acids. This behavior bears a certain resemblance to that of the triphenylmethane dyes.

2. Several of the factors involved in fixing the coloration of *Chromodoris* are considered in relation to the use of the intracellular indicator.

3. Protracted laboratory confinement of *Chromodoris* leads to apparent changes in the proportion of bound to dissolved (acid-sensitive) pigment in the skin, and also to alterations in the resistance of the tissue toward acids and other agents.

4. These observations afford an interpretation of variations found in measuring acid penetration in the case of variously pigmented *Chromodoris* tissues, and of the fact that freshly collected animals, uniformly colored, must be used to secure consistently comparable results.

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CELL PENETRATION BY ACIDS.

III. DATA ON SOME ADDITIONAL ACIDS.

By W. J. CROZIER.

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I. Several additional acids have been used in continuing a study of the penetration of living cells by acids, the object being to discover to what extent the visible penetration of diverse acids may be used to analyze the cell surface and the effect of various conditions upon it.[1] A description of the method according to which the indicator-containing tissue of the nudibranch *Chromodoris zebra* is utilized for this purpose has been given in a previous paper (Crozier, 1916). The present experiments having been made some months subsequent to those previously recorded, dilution curves were redetermined for acids (valeric, succinic) with which it was desired to compare the ones added to the list of eighteen first studied. In Table I the first and second series

TABLE I.

Penetration Time: A = First Series of Tests (Late Summer, 1915); B = Second Series (Spring, 1916). 27°C. (See Text.)

Dilution, normal.	Valeric.		Succinic.	
	A.	B.	A.	B.
liters	*min.*	*min.*	*min.*	*min.*
50	1.2	1.5	9.2	11.2
75	1.5	1.7		17.4
100	1.9	2.2	16.5	22.5
150	6.0	6.5		38
200	12.0	15.0	45	60
300	26.0	27.0		
400	55.0	62.0		

[1] I am indebted to Professor E. L. Mark for assistance in obtaining material for this work.

of measurements for succinic and isovaleric acids are compared; the figures represent in each instance the mean of ten or more concordant experiments. The differences to be observed are in the main due, I believe, to seasonal fluctuations in the condition of the tissue experimented upon.[2] The nature of this seasonal fluctuation may, however, be fairly simple; it is not improbable that the lower temperature of these animals during the winter months is responsible for the differences found. All the measurements recorded were carried out at 27°C., which corresponds pretty closely to the temperature of *Chromodoris* in summer time, while in winter their natural temperature is at times as low as 15°. The animals employed for this work were living at 19–20°, and it is not to be expected that a brief elevation to 27° would have the same effect upon them as would a long residence at that temperature. Variations in the alkalinity of the sea water may also be significant.

The acids used were *n*-caproic, maleic, and fumaric. The solutions were made up in distilled water, and (as in the previous measurements) in some instances also in rain water; in both cases the results were the same. This apparent disregard of familiar osmotic and other effects has been quite deliberate, since it is proposed to use these data as a starting point from which to begin a study of the actions of individual salts.[3] Distilled water (or rain water) in the absence of acid produces, of course, an almost (but not quite) immediate increase in permeability, which may be easily detected by subsequently placing the tissue in acid (see Table II); but acids, even in very dilute solution, lead (Osterhout, 1914) to a decrease of permeability, even toward themselves, which removes much of the objection to this procedure.

[2] It is perhaps necessary to state that the observed variations are not traceable to differences in the acid preparations. The temperature coefficients of acid diffusion will be considered in a subsequent paper.

[3] In this connection see Loeb and Cattell (1915) and Loeb (1915).

TABLE II.

The effect of preliminary treatment with distilled water upon the penetration time of acids. Typical data from one set of tests. Temperature = 24°C. The "control" gives the penetration time observed with similar tissue immersed directly in acid. There are added for comparison measurements with corresponding pieces of tissue killed by 1.0 per cent formaldehyde.

Animal.	In distilled H_2O.	Acid.	Time of penetration.	Control.	Dead tissue.
	min.		*min.*	*min.*	*min.*
A	3	0.05 N H_2SO_4.	2.5	5.0	2.0
	3	0.05 " "	2.3	5.1	1.8
B	2	0.1 N HCl.	1.5	1.8	0.9
	2	0.1 " "	1.3	2.1	1.2
	2	0.1 " "	1.5	2.1	1.1
				2.0	
C	1	0.1 N HCl.	2.1	2.0	1.0
	1	0.1 " "	2.0	2.5	1.0
	1	0.1 " "	3.2	1.9	
D	2	0.01 N valeric.	1.5	2.4	0.5
	2	0.01 " "	2.0	2.5	1.2
	2	0.01 " "	1.6	2.0	0.5

Table III contains a summary of the data on caproic, fumaric, and maleic acids, which are shown graphically in Fig. 1.

TABLE III.

The Penetration Time, Min., of Caproic, Maleic, and Fumaric Acids.

Acid.	Penetration time, 27 °C.										
Dilution	10.5	17.5	20	25	50	100	150	200	300	400	500
Caproic				2.3		4.7		9.3	17.0	25	60
Maleic	3.6		4.4		8.1	19.4		28.0		55	
Fumaric		4.6	5.4		13.0	27.6	40.0	55.0	80.0		

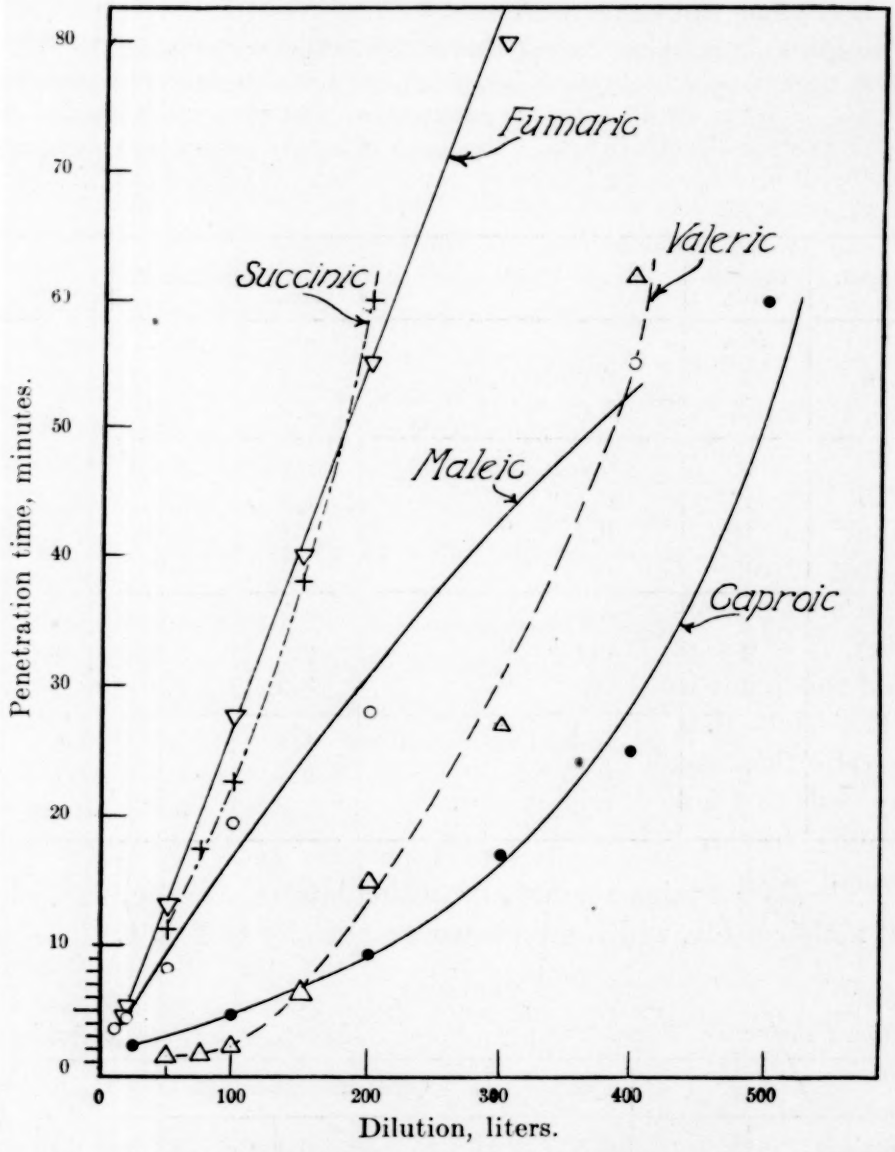

FIG. 1. Penetration of *Chromodoris* tissue. 27.0°.

II. The lipoid solubility of the monobasic fatty acids, aside from formic, follows the order shown in Table IV (quoted from Harvey, 1914, p. 468). This is precisely the order in which these acids, with the exception of caproic, had previously been found to affect the intracellular indicator of the *Chromodoris* tissues (Crozier, 1916). Caproic is now added to the list, and it will be noted that this acid also fits into the series (see Fig. 1).

TABLE IV.

Relative Lipoid Solubilities, Quoted from Harvey (1914).

Acid.	Caproic.	Valeric.	Butyric.	Propionic.	Acetic.
Partition coefficient. Xylol / water.....	3.5	0.6	0.1	0.02	0.00
Olive oil / water.....	7.25	1.78	0.44	0.14	0.05

The fact that in their first parts the curves for valeric and caproic acids are so near together, and even cross each other, does not interfere with this interpretation, since both are so far removed from the other weak fatty acids. The intracellular color change is an end result which involves several preliminary processes; one of these consists in the alteration of the cell surface to such an extent that the acid may enter; another consists in the diffusion of acid through the protoplasm. Provided the lipoid solubility of an acid be sufficiently great to speedily accomplish the former effect, it is not probable that an increase in lipoid solubility beyond this point would be markedly more efficient.

The view that this parallelism really indicates the method whereby these acids gain access to the interior of the integumentary cells of *Chromodoris* may be open to several qualifying objections, having reference to the variation of the partition coefficient with acid concentration, the nature of the lipoids possibly situated at the cell surface, and other similar points. There is, however, a correspondence between the penetration and solubility series, which is to a certain extent quantitative and cannot be ignored. Caproic and valeric acids are many times as soluble in olive oil or xylol as are the rest of the fatty acids, and they are similarly set apart from butyric, propionic, and acetic in the order of their cell penetrating ability. This is also true, in the same sense, for the concentrations of these acids necessary to reverse the phototropism of copepods (Loeb, 1911, p. 476). The combination of these acids with fatty substances situated at the cell surface enables the weak fatty acids to penetrate the cell in a sequence which has no reference to acid strength, either among themselves or in relation to other acids (compare Crozier, 1916).

III. Maleic and fumaric acids are of particular interest, because they may be closely compared with referenee to the rôle

of ionization in determining cell penetrating power. Harvey (1914) found that from 0.01 N solutions fumaric acid required 12 to 15 minutes, maleic acid 20 minutes, to penetrate the testis epithelium of *Stichopus ananus*. With this sequence my values (27.6 and 19.4 minutes, respectively) do not agree.

The near position of the curves for succinic, maleic, and fumaric acids, having respectively the ionization constants 0.00665, 1.17, and 0.093, is sufficient in itself to show that ionization is not the sole determinant of penetrating ability. Yet the order maleic > fumaric, succinic bears out the conclusion previously arrived at (Crozier, 1916) that, when closely related acids are considered, their sequence in affecting the intracellular indicator is controlled by acid strength, provided the ionization is neither too high nor too low.

SUMMARY.

IV. The penetrating ability of caproic, maleic, and fumaric acids has been tested for the indicator-containing tissue of the nudibranch *Chromodoris zebra*. The positions of these acids with relation to the eighteen previously studied are such as to support the conclusion derived from the latter; namely, that for any given acid at least two factors determine its relative position in the penetration series. One of these influences is ionization, the second concerns more particularly the union of the acid with one of the several constituents of the cell surface. In the case of the weak monobasic fatty acids, this constituent is of a fatty nature.

AGAR'S ISLAND, BERMUDA.

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FEEDING EXPERIMENTS ON THE SUBSTITUTION OF PROTEIN BY DEFINITE MIXTURES OF ISOLATED AMINO-ACIDS.[1]

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Investigations concerned with the question of the indispensability of amino-acids to the animal organism and with their precise significance in metabolism, have been pursued notably along two general lines. The first line of investigation, although followed by several workers independently with varying results, has been perfected by Abderhalden and has given the most fruitful results in his hands. The method is based upon the fact, which has been repeatedly proven experimentally, that the animal organism can be maintained in equilibrium and can even exhibit considerable increases in weight and nitrogen when the nitrogenous requirements are covered by preparations obtained by hydrolyzing protein material with enzymes until no positive biuret test can be secured, when presumably only amino-acids and probably the simpler polypeptides were present. One of the most successful experiments of this nature was recently reported by Abderhalden.[2] In this experiment a dog was kept for 100 days on a hydrolyzed meat preparation (ereptone) supplemented by non-nitrogenous nutrients. During this time the animal behaved normally, seemed in perfect health, and increased in weight from 13.75 to 23.10 kg. Similar successful experiments in which completely

[1] The results presented in this paper formed part of a thesis submitted by the author to the Graduate School of the University of Illinois in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

[2] Abderhalden, E., *Z. physiol. Chem.*, 1913, lxxxiii, 444.

hydrolyzed preparations[3] were used during some of the periods have been still more recently reported by the same investigator,[4] using dogs and rats as experimental subjects.

On the basis of such results the essence of Abderhalden's method was to remove certain amino-acids from the unknown mixture of the products of protein hydrolysis as completely as available methods would permit, and to determine the nutritive value of the residual mixture of amino-acids by testing its ability to maintain body weight and nitrogen equilibrium when fed to experimental animals in an otherwise adequate ration as the sole source of nitrogen. If the residual mixture proved itself of equal nutritive value to the original, the conclusion seemed justified that the amino-acid or amino-acids removed were not indispensable to the body, but could be synthesized from other compounds, provided, of course, the removal was reasonably complete. If the residual mixture of amino-acids was clearly inadequate to cover the nitrogen requirements, the proof of the indispensability of the amino-acid or amino-acids removed was completed by adding again to the ration the substances removed (isolated in a pure condition) and thus restoring its complete nutritive value. In cases in which the mixture was still inadequate after such addition, no conclusions would be justified. Such ambiguous results might be produced, for example, by the removal of substances other than those intentionally removed, by the incomplete removal of reagents, such as mercury salts, which may exert a harmful effect on the animal organism, or by chemical changes in the residual amino-acids incidental to the removal of those under investigation.

In Abderhalden's laboratory this method has been applied with striking success in many instances. The inability of the body to synthesize tryptophane and tyrosine has been repeatedly and clearly shown. In these experiments the tryptophane was removed according to the method of Hopkins and Cole with mercuric sulfate in 5 per cent sulfuric acid solution, and the tyrosine

[3] The completeness of hydrolysis in these cases was determined by amino nitrogen determinations before and after boiling with concentrated mineral acid. These preparations were submitted first to the prolonged action of gastric, pancreatic, and intestinal juices or extracts and were then digested for 8 to 10 hours on the water bath with dilute sulfuric acid.

[4] Abderhalden, *Z. physiol. Chem.*, 1915, xcvi, 1.

by concentration of the neutralized solution *in vacuo*. The addition of tryptophane or tyrosine, respectively, to the residual mixtures in most experiments had a distinct effect in correcting their nutritive deficiencies entirely, as indicated by the maintenance of body weight of the experimental animals to which the mixtures were fed, and the restoration of a positive balance of nitrogen. Furthermore, in his more recent experiments Abderhalden has shown that tyrosine cannot be substituted by the corresponding keto acid, oxyphenyl pyruvic acid, or by phenyl pyruvic acid, nor could it be substituted satisfactorily in most experiments by phenylalanine, in accordance with the work of Embden and Baldes.[5] Some improvement, however, in nitrogen equilibrium was noted upon the addition of phenylalanine to the tyrosine-free ration, suggesting possibly a partial oxidation of this amino-acid to tyrosine.

The work on the nutritive value of tryptophane and tyrosine reviewed above embodies the only entirely successful results obtained with the method. Attempts to determine the nutritive value of proline and cystine have not led to results sufficiently clear cut to warrant final conclusions, while still less encouraging are the experiments to test the diamino-acids, by removal according to the procedure of Kossel and Kutscher.

Henriques and Hansen[6] have published the results of an experiment similar in character to the work of Abderhalden, which led them to conclude that the diamino-acids could be entirely dispensed with by the animal body. The experiment was performed on a rat and the ration contained, besides non-nitrogenous constituents, a mixture of the products of protein hydrolysis containing presumably only monoamino-acids. It was prepared from an enzyme digest of a protein by precipitation with phosphotungstic acid, filtration, removal of excess reagent from the filtrate, and evaporation to dryness *in vacuo*. Unfortunately the particular protein used was not mentioned, and the lack of all details of the phosphotungstic acid precipitation precludes any attempt to judge of the completeness of separation of monoamino- from diamino-acids. The experiment lasted only 26 days. During the last 17 days only were positive nitrogen balances obtained and they

[5] Embden, G., and Baldes, K., *Biochem. Z.*, 1913, lv, 301.

[6] Henriques, V., and Hansen, C., *Z. physiol. Chem.*, 1904–05, xliii, 417.

were such that they could hardly be said to constitute convincing evidence of the nutritive adequacy of the ration fed, especially in view of the fact[7] that the determination of the total urinary nitrogen per day of rats, with the exercise of the utmost care as to collection and preservation, is subject to errors of 10 per cent or more, due to incomplete collection. The body weight increased slightly during the last half of the experiment, but during the last 3 days it declined slightly but consistently. It is unfortunate that the experiment was not continued further to determine whether this final decrease was significant or not. This criticism is especially justified by the many experiments that may be quoted on the feeding of synthetic rations, in which entirely erroneous conclusions may be drawn if attention is confined to the first 20 or 30 days of observation.

The second general method of investigation of the nutritive significance of the amino-acids to the animal organism is associated more especially with the names of Osborne and Mendel and their associates, and has given by far the most valuable results in their hands. The method consists essentially in the feeding of isolated and carefully purified proteins, obtained from a wide variety of sources, incorporated in basal rations otherwise adequate for normal growth or maintenance and composed of artificial food materials, in most part of known composition and containing at most but minimal percentages of nitrogen or protein. By this method different proteins have been clearly shown to have different values in nutrition, in one respect or another, and these differences have been traced with great success to differences in amino-acid content. Thus, the significance of some of the amino-acids has been made evident, either by noting the peculiar effect attendant upon their absence in proteins or their occurrence in minute amounts, or by observing the effects produced upon supplementing proteins by their addition.

Of the results obtained by Osborne and Mendel with this second method of investigation the most general biological significance is attached to the demonstration that lysine is essential for growth. Gliadin, the alcohol-soluble protein of wheat, when fed

[7] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 156*, 1911, pt. i, 12.

to rats as the sole protein in a ration, is capable of maintaining an animal for long periods of time at constant weight, but is not able to induce normal growth. A slow growth would often result, while at other times only maintenance was secured. This characteristic stunting with gliadin resulted even when the calorie intake was sufficient for optimum growth, and has been successfully traced[8] to the low content of this protein in lysine, since upon the addition of lysine to the ration, normal growth could be secured.

Zein, the alcohol-soluble protein of the corn kernel, has been shown by Osborne and Mendel (and others before them) to be unable to support either growth or maintenance. Upon analysis, this protein has been shown to lack the amino-acids glycocoll, tryptophane, and lysine. The addition of tryptophane to the protein renders it adequate for long continued maintenance,[9] the further addition of lysine renders it adequate for fairly normal growth,[9] but for normal growth a further addition of arginine, which is present only in small amount in zein, appears to be necessary.[10]

Recently, in an investigation[11] to determine the effect of different percentages of various proteins in the ration on the growth of white rats, Osborne and Mendel have shown that rations containing percentages of casein inadequate for normal growth may be rendered adequate by the addition of the sulfur-containing amino-acid cystine to the ration. These results are intelligible in view of the comparatively low cystine content of casein.[12]

In properly appraising the two general lines of investigation of the nutritive value of amino-acids briefly described—on the one hand, that method associated mainly with the name of Abderhalden, and, on the other hand, that exemplified best by the work of Osborne and Mendel—it must be granted that they have contrib-

[8] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 332.

[9] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 378.

[10] Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 11.

[11] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351.

[12] Whether the favorable influence of cystine when added to rations containing 9 per cent or less of casein was due to its sulfur content entirely and could be duplicated by other available sulfur-bearing compounds is open to question. It is to be noted that the ration as a whole, as well as the casein, was poor in sulfur.

uted much valuable information. Our conceptions of protein metabolism and protein requirements must be profoundly modified as a result of this pioneer work. The work of Osborne and Mendel, in particular, is worthy of the highest commendation. By working with small experimental animals and using artificially prepared rations, consisting in large part of carefully purified substances of definite composition, they have succeeded in performing numerous experiments under carefully controlled conditions. The results have been clear cut and unequivocal in most instances. However, no criticism of these methods would be complete without due account being taken of their limitations.

The limitations of the Abderhalden method are obvious. It is only applicable to those amino-acids that can be readily and quantitatively isolated (or practically so) from protein hydrolysates, and isolated by means of reagents that can subsequently be entirely removed from the residual mixture. Furthermore it is essential to the success of the method that the residual amino-acids are not changed or destroyed during the manipulations involved in the removal of the amino-acid or acids to be tested, nor by the reagents employed for this purpose. In view of the difficulty, if not the impossibility, of conforming to the above conditions in the removal of the large majority of the amino-acids from mixtures obtained from the hydrolysis of proteins, this method is seriously restricted in its usefulness. It seems probable, in view of the methods available for the quantitative separation of amino-acids, that the Abderhalden procedure cannot be of any lasting value without at the same time becoming so cumbersome as to be of little practical utility.

The limitations of the Osborne and Mendel method are also obvious. Its success depends upon a knowledge of the amino-acid content of the different proteins investigated. The extremely unsatisfactory condition of this knowledge as regards the large majority of the proteins studied needs no comment. The most striking results have been afforded by a study of the nutritive value of proteins totally or largely deficient in one or more amino-acids. But such proteins are few in number, apparently, and their characteristic effects may involve some peculiar proportions among those amino-acids that do occur in them, proportions which cannot be intelligently modified in the absence of reliable data as

to their precise nature. A lack of such data, in the case of phaseolin for example,[13] has blocked any attempt to determine the cause of its inadequacy for either maintenance or growth. A similar situation exists in connection with the poor nutritive value for growth of many of the proteins of the leguminous seeds, such as vignin.[14]

Any intelligent supplementing of proteins with isolated amino-acids is also impossible in the absence of at least fairly reliable information of their amino-acid content. Thus Osborne and Mendel state[15] that they did not attempt to supplement lactalbumin with purified amino-acids to determine whether normal growth could be secured with less than 9 per cent of this protein in the ration, on account of inadequate information as to its amino-acid make-up.

However, even though a good knowledge of the amino-acid content of proteins were available, due to the evolution of accurate analytical methods, a study of the nutritive value of native proteins and of native proteins supplemented by amino-acids can hardly be expected to reveal the nutritive values of each individual amino-acid, nor to determine whether each amino-acid may or may not be synthesized by the animal organism. Much less could such a method of study be expected to yield complete information as to quantitative amino-acid requirements. The impossibility of such attainments by the use of the Osborne and Mendel method rests in the impossibility of controlling the amino-acid intake absolutely and of experimenting with any desired combination of amino-acids. For example, the suggestive theory of Osborne and Mendel[16] to the effect that the only reason for the destruction of any protein at all in maintenance might be to liberate a small amount of one or two, or at most a relatively small number of amino-acids, to engage in some "hormone-like physiological duty upon which proper metabolism might depend," obviously could not be tested by their method of experimentation.

Thus while it would be hazardous to attempt to predict to what extent scientific ingenuity will overcome the obstacles inherently

[13] Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 9.
[14] Osborne and Mendel, *Z. physiol. Chem.*, 1912, lxxx, 316.
[15] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 356.
[16] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 328 and 329.

limiting the applicability of this method to the problems of amino-acid synthesis in the animal organism and of amino-acid requirements, it may be confidently asserted that only a very incomplete solution can be expected from even the most extensive experimentation with it.

The most direct method of attacking these problems, and the only method with unlimited possibilities, is the feeding of definite mixtures of isolated amino-acids, to the complete exclusion of proteins and all of their intermediary degradation products. It is the main purpose of this investigation to test the feasibility of this method of experimentation.

Several attempts to cover the nitrogenous requirements of dogs by substituting the protein of a ration by a mixture of purified amino-acids have been made by Abderhalden.[17] After many unsuccessful trials one experiment of 8 days' duration is reported in which the most encouraging results were obtained. The difficulties encountered in the previous trials were, first, inducing the animals to consume the rations containing amino-acids, and, second, avoiding the digestive disturbances which such rations were especially liable to produce, such as vomiting and diarrhea. In the 8 day experiment above mentioned a mixture of isolated amino-acids in their naturally occurring forms, containing all of the amino-acids commonly obtained in the hydrolysis of proteins with the exception of oxyproline, was fed. Each day's ration consisted of 25 gm. of amino-acids, 50 gm. of a glycerol-fatty acid mixture, 2 gm. of cholesterol, 50 gm. of glucose, and 5 gm. of bone ash, the amino-acids constituting about 9 per cent of the total ration. Though the ration possessed no decided taste, it was a matter of great difficulty to induce the animal to consume it. It was necessary to feed the dog often with small portions of the food. During the 8 days of feeding the body weight was constant. A negative nitrogen balance was obtained on the 4th, 5th, 6th, and 8th days, though the average balance of the period was positive. During the subsequent 7 day period, when 25 gm. of hydrolyzed beef (with a smaller percentage of nitrogen) were substituted for the amino-acid mixture in the above ration, the body weight increased slightly and a much more favorable nitrogen balance was secured.

Another dog was fed for 6 days with a ration containing 22.1 gm. of the same amino-acid mixture, 2 gm. of hydrolyzed nucleic acid, 25 gm. of fat, 20 gm. of sucrose, 20 gm. of glucose, and 5 gm. of bone ash. The weight of the dog was practically constant throughout the period, though negative nitrogen balances were obtained on the 3rd, 4th, and 5th days of feeding. The experiment was terminated by the refusal of the dog to consume any more of the ration. Substitution of the amino-acid mixture by hydrolyzed meat in the above ration was followed by more favorable results, as in the preceding experiment.

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[17] Abderhalden, *Z. physiol. Chem.*, 1912, lxxvii, 22.

Abderhalden concludes from these two feeding periods that no physiologically indispensable amino-acid was absent from the mixture fed. This conclusion, however, must be considerably discounted by reason of the shortness of the periods of amino-acid feeding, and by the unsatisfactory nitrogen balances obtained. The objections inherent in such short feeding periods as occur throughout Abderhalden's investigations need no comment in view of the frequent experience, especially of American investigators, with rations that appear to be adequate for long periods of time, but that ultimately lead to nutritive failure. Quoting a significant remark in one of the papers of Osborne and Mendel:[18]

"If we had been content to discontinue the experiments after a reasonable period, many of the declines [in body weight] evidently associated with imperfections in the dietary and readily checked by a change in feeding, would have escaped attention."

Osborne and Mendel[19] have recently reported briefly an attempt to cover the maintenance nitrogenous requirements of rats by a mixture of tryptophane, cystine, histidine, tyrosine, phenylalanine, proline, and ammonium citrate or urea. In their own words:

"All such attempts failed, even when the supply of energy in the form of non-protein substances was liberal and the food contained all of the necessary inorganic salts and 'food accessories,' and in addition at least 0.5 per cent of protein, present in the 'protein-free' milk. On such diets the rats declined just as rapidly as when the amino-acid additions were not made."

It is evident from this review of the few experiments concerned with the feeding of mixtures of amino-acids to the exclusion of

[18] Osborne and Mendel, *J. Biol. Chem.*, 1912–13, xiii, 245 and 246. Further evidence of the unreliability of short feeding experiments is afforded by the work of Dr. Ruth Wheeler. According to this investigator (*J. Exp. Zool.*, 1913, xv, 210):

"Animals have a surprising power of getting along for a time on a qualitatively inadequate diet. Mice kept upon a ration in which two-thirds of the protein was gelatin and the other third casein did not show a decline in body weight for 21 days, although the ultimate decline of every animal fed upon this food showed it to be insufficient; another group made material gains in weight for 17 days on a diet upon which all ultimately lost weight rapidly and died unless the food was changed."

[19] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxv, 2.

protein that no conclusive results have thus far been obtained; that is, that no definite mixture of isolated amino-acids has been shown to be capable of supporting life indefinitely. With the purpose of discovering some such mixture, the experimental work reported below was undertaken. The results secured to date have not been entirely successful, but several points of interest have been observed that seemed of sufficient importance to warrant publication.

EXPERIMENTAL INVESTIGATION.

The Selection of Experimental Animals.—The arguments in favor of the use of the smaller experimental animals in nutrition work have been well summarized by Osborne and Mendel:[20]

"The preparation of suitable pure food supplies on a scale sufficient for long periods and in economically procurable amounts is thereby rendered possible. The necessary scientific measurements and analyses can be conducted on a scale impossible for larger animals, and the problem of care and attention is equally simplified. Experiments can be duplicated without great effort and individual peculiarities eliminated by force of numbers. Furthermore, the various stages of growth and maturity are completed in the smaller animals within relatively short periods of time, so that the permanent effects of any dietary become apparent within a range of days or months that is not outside of ordinary experimental possibilities of observation. As illustrations of some of these features it may be recalled that the ultimate effects of complete inanition are apparent in 4 or 5 days[21] in rats or mice; in dogs the fatal outcome may be delayed for many weeks. The duration of life in the white rat is about 3 years; about 280 days suffice to complete the entire period of growth to maturity."

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[20] Osborne and Mendel, *Carnegie Institution of Washington, Publication No. 156*, 1911, pt i, 6.

[21] In the course of one of our feeding experiments with white mice we attempted to feed an amino-acid ration to a female mouse weighing 23.6 gm. For the first 4 days the food was untouched; on the 5th day, 1.5 gm. of ration were eaten. For the next 2 days the food was untouched and it was withdrawn on the 7th day, from which time the animal received water only. The body weight rapidly declined, though aside from emaciation no other symptoms of malnutrition, such as roughened coat, infected eyes, etc., were apparent. On the 15th day of observation, *i.e.*, 10 days after the 1.5 gm. portion of food was eaten, the animal died at a weight of 11.0 gm., having lost over 53 per cent of its initial weight. From this observation it would appear that the resistance of mice to inanition is much greater than is indicated in the quotation cited.

In the experimental project reported below the main limiting factor was the amounts of amino-acids that could be bought or prepared. It was, therefore, considered expedient to use mice or rats for the work, and of these two species of animals mice were considered more satisfactory, since they are only about one-tenth as heavy as rats of equal age and consequently they require a much smaller amount of food for maintenance and growth. Their span of life is also shorter than that of the rat. It has been stated that the average life of a white mouse is less than 2 years, that they are sexually mature at 2 months of age, and are fully grown at 150 days or even less. It would therefore appear that the effect of any inadequacy in a ration would be manifested in an even shorter period of time with mice than with rats.

It is certainly of some importance to consider the suitability of mice (or rats) as experimental subjects, not only from the standpoint of experimental convenience and expediency, but also from the standpoint of the biological significance of any experimental results obtained. It is of no economic importance or of no human interest simply to determine the qualitative or quantitative food requirements of such animals as rats or mice, if such requirements are not indicative, in a general way at least, of the food requirements of all mammalian organisms.

One is apt to have the impression, probably largely on account of the great discrepancy in size between rats and mice and animals of any economic or other importance to man (including man himself), that these smaller experimental animals are inherently unsuited for investigation purposes, because any results obtained with them must possess a very limited significance. In opposition to such an impression stands the work of Folin and Morris[22] on the great similarity in the composition of the urine and also in purine metabolism between the rat and man. The extensive investigations of Donaldson[23] on the growth of the white rat have revealed close similarities to man, the similarity extending even to the growth of the brain and spinal cord. The close parallelism between the results that Hart and McCollum[24] have obtained

[22] Folin, O., and Morris, J. L., *J. Biol. Chem.*, 1913, xiv, 509.

[23] Donaldson, H. H., Boas Memorial Volume, New York, 1906, 5–26; also *J. Comp. Neurol. and Psychol.*, 1908, xviii, 345.

[24] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.

with pigs and with white rats in their study of the influence of restricted rations on growth also testifes to the general biological significance of experimental results secured with rats and presumably, also, of those secured with mice.

Such concrete illustrations of the close biological relations existing between the different species of mammals lend support to the general practice among physiologists and investigators in nutrition and related fields—a practice that is perhaps too generally accepted and too seldom submitted to critical scrutiny—to attach a general biological significance to experimental results obtained upon one particular species, or more truly upon a few representatives of one particular species of animals. The main justification of this practice is that, though no extensive systematic investigations on the comparative physiology and metabolism of the different species of mammals have been attempted, the experimental findings upon a large variety of animals are all remarkably consistent with one another, if not actually confirmatory of one another. Those differences that have been found in the metabolism of different species of mammals are largely quantitative and not qualitative. Therefore it has been concluded that experimental results obtained with one species of mammals may be considered as at least qualitatively applicable to mammals in general. Obviously accurate observations on rats or mice should be considered as valuable, in their general applicability, as observations on any other species of animal.

Experimental Details.—The mice were confined in galvanized iron cages, 8 inches in diameter and 6 inches high. In most of the experiments two mice were confined in a single cage. A small amount of paper excelsior[25] was always kept in the cages.

The rations were placed in small porcelain crucibles, $1\frac{3}{4}$ inches in diameter at the top and about $1\frac{1}{8}$ inches in height, which were wired to the sides of the cages. To prevent undue scattering of the food, the top of the crucible was fitted with a flanged ring of tinned copper, the hole in the ring being about 1 inch in diameter. This was crossed by two wires, also of tinned copper, at right angles to each other, so that there were four holes through which the mice could insert only their heads. With this device it was

[25] The paper excelsior used in all of the work analyzed 0.80 per cent ash and 0.05 to 0.06 per cent nitrogen.

hoped that all scattering of the rations would be avoided, but almost invariably with the amino-acid rations considerable scattering would occur in spite of all precautions. In such cases, to insure a fairly accurate determination of the food intake, the cage was placed over a crystallizing dish of such size as to fit under it tightly. This prevented the inroads of any wild mice. To insure a good separation of food waste and urine, a piece of heavy filter paper about 4 inches square was placed immediately under the food crucible in the crystallizing dish. With these arrangements it was rare that a good determination of the food intake could not be made, though only at the expense of much time and patience. The food was weighed out once a day, at which time the uneaten food from the preceding day was determined. The mice were weighed once a day in the amino-acid experiments, and once every other day in the experiments with other rations.

Water was supplied to the mice from an inverted bottle (attached to the removable top of the cage) provided with a bent delivery tube protruding a few inches into the cage. This method is very satisfactory, if care is taken to insure that the delivery tubes are always full of water. This depends primarily on the bore of the tube, the angle at which it is bent, and the size of the opening at the end of the tube, which is necessarily constricted somewhat to prevent undue loss of water. Distilled water only was used.

The mice were kept in light and dry quarters. During the autumn months when no artificial heat was available, the temperature occasionally fell to 65° or even 60° F., but later after the installation of a radiator, the temperature always remained above 70° and was generally maintained between 75° and 80°. In the hottest days of summer the temperature often rose above 90° F., but at such times conditions were alleviated somewhat by the use of an electric fan.

The cages were cleaned out once a day generally, and in all cases at least once every other day. They were sterilized whenever such measures were required (generally once in 1 or 2 weeks) by passage through a hot solution of strong alkali and thorough rinsing with water.

The amino-acids used in the feeding experiments were mostly prepared from protein material. The tyrosine, cystine, glutamic

acid, lysine, histidine, and arginine were prepared in the usual way from various proteins after complete acid hydrolysis. The tryptophane was prepared from pancreatine digests of casein by the method of Hopkins and Cole. The valine was synthesized, and the aspartic acid, asparagine, leucine, alanine, glycocoll, isoleucine, and phenylalanine were bought on the market and were in most cases Kahlbaum products. The *l*-proline used in some of the rations was an impure product obtained by alcohol extraction of protein hydrolysates with acid and subsequent isolation of the copper salt. Oxyproline and serine were not available for any of this work.

Most of the amino-acids prepared in the laboratory were not of tested purity. The tyrosine and cystine used were found to contain very close to the theoretical percentage of nitrogen and one tryptophane preparation was likewise found to be a very good product as judged by this criterion.

Basal Rations for Maintenance.—In view of the fact that growth probably sets a higher standard as regards amino-acid requirements than does simple maintenance, the logical point of attack in this investigation appeared to be the amino-acid requirements for maintenance rather than for growth. Another argument in favor of this procedure was that adult mice, which would be used in maintenance experiments, could probably withstand the digestive disturbances anticipated in the feeding of amino-acid rations better than immature animals.

It was therefore necessary to decide upon a suitable basal ration for maintenance made up of food substances containing minimal amounts of nitrogen. The rations used by McCollum and Davis, consisting of dextrin, lactose, purified butter fat, agar-agar, and a synthetic salt mixture, besides protein, for which mixtures of amino-acids could be substituted in our experiments, seemed ideal for the purpose. Unfortunately we could not secure long continued maintenance of mice with such a ration, even when casein was used to cover the nitrogen requirements, the trouble seeming to rest with the salt mixture. The ration of Osborne and Mendel, consisting of protein 18 per cent, "protein-free milk" 28 per cent, starch 26 per cent, lard 10 per cent, and purified butter fat 18 per cent, was found to be capable of maintaining

mice for long periods of time, thus confirming the results of Wheeler.[18, 26]

The main objection to such a ration in an experiment of this nature is the fact that the "protein-free milk" as prepared according to the directions of Osborne and Mendel always contains protein nitrogen. In attempting to obviate this difficulty we have slightly modified the method of preparing this product.[27] After precipitating the casein from milk by a slight excess of HCl, filtering off the coagulum, boiling for ½ minute to coagulate the lactalbumin, and filtering off this precipitate, the solution was carefully neutralized to litmus with NaOH and again filtered, the filtrate being then evaporated to a thick paste on the water bath at 70°C. and dried in a current of warm air. The last filtration, which constitutes the modification introduced, removes about 0.1 per cent of nitrogen, presumably protein nitrogen, figured on the basis of the dried product, or one-seventh of the total nitrogen of the first filtrate. Some of the mineral salts are also removed, though the nutritive value of the preparation for maintenance does not seem to be impaired at all if it constitutes as much as 28 per cent of the ration. This modified preparation of "protein-free milk" was used in many of the feeding experiments reported below.

Feeding Experiments with Mixtures of Amino-Acids.—In the first attempts to cover the nitrogenous requirements of mice by defi-

[26] While the Osborne and Mendel casein and "protein-free milk" ration appears to be satisfactory for the maintenance of weight and health of white mice, it does not appear to be satisfactory for the production of offspring. Comparatively few cases of pregnancy were observed with mice on this ration. Where pregnancy occurred birth was generally premature, apparently, and the young were generally eaten by the mother within 24 or 48 hours. In the course of over a year's experience only three young (out of a litter of five) were successfully raised until weaning, on this ration, and in these cases growth during the nursing period was noticeably slower than normal.

[27] Mitchell, H. H., and Nelson, R. A., *J. Biol. Chem.*, 1915, xxiii, 459. It might be said that practically all of the investigation work reported in this paper was completed before the trichloroacetic acid method of preparing protein-free milk was elaborated. This method, while probably better suited to the purposes of this work than any other, was not used in the preparation of any of the rations tested.

nite mixtures of amino-acids, the Osborne-Mendel ration was modified simply by the substitution of the 18 per cent casein by 18 per cent of definite mixtures of amino-acids, containing fifteen or fewer of the seventeen amino-acids commonly found in proteins, the two amino-acids lacking being serine and oxyproline. The proportions of the individual amino-acids included in these mixtures were more or less arbitrarily chosen, since there is little information at hand that would logically furnish a presumption in favor of any one mixture as opposed to others. In preparing these (as well as other mixtures tested) some consideration was given to the available analyses of pure proteins known to be complete for maintenance and growth; more consideration was necessarily paid to the stock of amino-acids on hand.

The results of the feeding experiments with 18 per cent of amino-acids were not successful and will not be given in detail. In all cases a decline in weight began as soon as the amino-acid rations were given, and in all cases the food consumed per day was light, especially as the experiment continued, so that it cannot be said that the failure to maintain weight was due to an incomplete amino-acid intake any more than to an incomplete energy intake. In several cases the mice on these amino-acid rations died within 2 weeks, and in other cases after longer periods. Four of the mice that died showed symptoms of severe diarrhea. The other six mice that died on the amino-acid rations showed no outward symptoms of digestive disturbance. No postmortem examinations of these mice were made.

The most successful of these preliminary experiments were run on two male and two female mice. The former two mice weighed from 20 to 21 gm. at the beginning of the experiment and were kept on rations containing mixtures of the 15 amino-acids available, for 27 days. At this time they had lost 37 and 45 per cent of their original weight. The two female mice weighed from 18 to 18.5 gm. initially and were kept on an amino-acid ration for 28 days, at which time they had lost 37 and 43 per cent of their weight. The condition of these four mice at the end of 27 and 28 days was obviously poor. Their hair was rough, emaciation was extreme, and they were so weak that they could barely use their hind legs. Upon changing to a ration containing 18 per cent of casein, recovery of the original weight occurred slowly in three

cases. In one case recovery was not complete, though the health and condition of the animal was apparently restored.

At this stage of the investigation the publications of Osborne and Mendel[11] and of McCollum and Davis[28] on minimum protein requirements appeared, in which it was shown that the protein requirements for the maintenance of rats may be covered by rations containing as low as 3 or even 2 per cent of protein. A plan for further work with the amino-acid rations immediately suggested itself. By lowering the percentage of amino-acids in the ration it was hoped that the food intake of the mice could be raised to normal and thus assure a fair test of the amino-acid mixtures, provided that the diminished consumption of the 18 per cent amino-acid rations was due simply to some disagreeable flavor imparted to the rations by the amino-acids contained in them.

This plan was first tested with a ration containing 8.1 per cent of a mixture of six amino-acids:

	per cent
Tyrosine	1.0
Cystine	1.0
Glutamic acid	4.0
Tryptophane	0.6
Histidine	0.5
Arginine	1.0

This ration was given to a male mouse with an initial weight of 21.4 gm. For the first 9 days the food intake was adequate, apparently, in covering the energy requirements, being from 2 to 3 gm. per day. The body weight decreased steadily, however, probably due to an inadequate amino-acid intake. On the 9th day the mouse weighed only 15.6 gm. From the 9th through the 50th day of the experiment the amino-acid ration was alternated in an irregular fashion with a non-nitrogenous ration, containing neither protein nor amino-acids except in so far as these are contained in "protein-free milk."

The introduction of the non-nitrogenous ration exerted a decidedly favorable influence on the body weight, which showed no tendency to decrease during the next 16 days of observation—on the 25th day of observation the weight of the mouse was 15.2

[28] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 415.

gm. However, from here on, a slow decline ensued. During the period of mixed feeding the food consumption was much better on the non-nitrogenous than on the amino-acid rations, and almost invariably a day's feeding of the former when immediately preceded by a day's feeding of the latter ration produced a more or less clearly marked increase in body weight. For 50 days this mouse was kept alive on rations containing no appreciable amount of protein. Towards the last of the test the animal was in very poor condition and weighed 11.4 gm. on the 50th day. An attempt at recuperation on rations containing small amounts of casein (2 to 3 per cent) supplemented with cystine and tryptophane failed, and the mouse died on the 58th day of the experiment at a weight of 10.2 gm. The total loss in weight amounted to about 52 per cent of the initial weight.

A somewhat similar experiment to that just described was undertaken with a male and female mouse each weighing initially about 23 gm. For the first 9 days of the experiment these mice were put upon a non-nitrogenous ration and they declined steadily and rapidly in weight. From the 9th to the 40th days non-nitrogenous and amino-acid rations were fed alternately, as in the preceding experiment. The decline in weight still continued, but at a distinctly more moderate rate, though the food consumption was not noticeably increased, decreasing slowly, in fact, both absolutely and per 100 gm. of body weight.

These two experiments showed that the body weight curves of mice on amino-acid rations could be favorably affected by interposing non-nitrogenous rations alternately with the test rations. This favorable effect may be due simply to the fact that the former rations were consumed in larger quantities than the latter so that the energy requirements could be covered, or at least more nearly covered, than on amino-acid rations entirely. While this régime at the same time lowers the intake of amino-acids, it might still be consistent with nitrogen equilibrium in view of the known low protein requirements for maintenance of rats, and presumably also of mice. Therefore a series of feeding experiments were planned to test the value of various amino-acid mixtures which were included in percentages ranging from 2.5 to 9 in rations containing 28 per cent of "protein-free milk" (in some cases the modified preparation described above), 26 per cent starch, 10 per cent

lard, 18 per cent butter fat, and sufficient sucrose to make the ration up to 100 per cent. These amino-acid rations were fed alternately with a non-nitrogenous ration consisting of 10 per cent sucrose, 34 per cent starch, 28 per cent "protein-free milk" (in most cases the modified preparation), 10 per cent lard, and 18 per cent butter fat. A few of the more successful of these experiments, the results of which are represented graphically in Charts I to V, will now be considered.

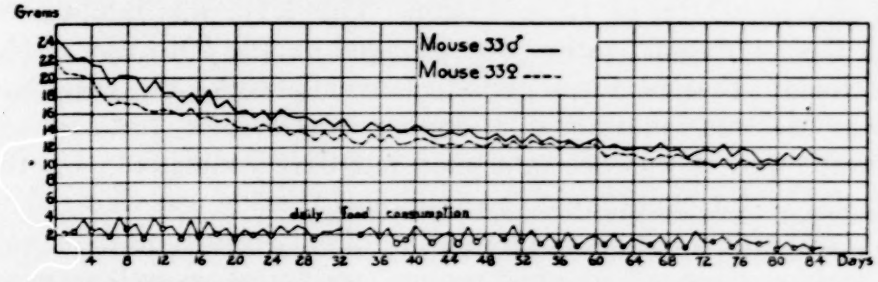

CHART I. The upper two curves represent the changes in body weight of Mice 33 ♂ and 33 ♀. The circled points on the lower curve, representing the daily food consumption of the mice, indicate the days on which amino-acid rations were consumed. On the other days practically non-nitrogenous rations were given. For the period up to and including the 59th day the amino-acid ration used contained: 0.20 per cent histidine, 0.25 per cent glycocoll, alanine, valine, isoleucine, phenylalanine, proline, and aspartic acid, 0.30 per cent tryptophane, 0.37 per cent cystine, 0.40 per cent arginine and lysine, 0.45 per cent asparagine, 0.50 per cent leucine and tyrosine, and 0.75 per cent glutamic acid—a total of 5.62 per cent of amino-acids. This ration contained the most complete amino-acid mixture used in all the experiments reported; *i.e.*, fifteen acids. Of the amino-acids commonly occurring in proteins only serine and oxyproline are not represented. From the 60th to the 76th days inclusive the amino-acid ration used contained: 0.25 per cent isoleucine, cystine, tryptophane, arginine, histidine, valine, aspartic acid, and phenylalanine, 0.50 per cent tyrosine and leucine, and 1.00 per cent glutamic acid—a total of eleven amino-acids, making up 4.00 per cent of the ration. From the 77th day to the end of the experiment the amino-acid ration used contained ten amino-acids: 0.25 per cent cystine, tryptophane, valine, histidine, and phenylalanine, 0.50 per cent tyrosine, leucine, arginine, and asparagine, and 0.75 per cent glutamic acid—a total of 4.00 per cent.

In Chart I are recorded the results of a feeding experiment on two mice that were fed alternately, as indicated by the curve at the bottom of the figure representing the daily food consumption, a non-nitrogenous ration and an amino-acid ration containing

5.62 per cent of a mixture of fifteen amino-acids—the most complete amino-acid mixture tested in this investigation. Of the amino-acids commonly reported as occurring in proteins, this mixture lacked only serine and oxyproline. This system of feeding was continued for 59 days, during which period Mouse 33 ♂ decreased very gradually in weight from 24.4 to 12.6 gm., a total loss of over 48 per cent of the original weight. In the same period Mouse 33 ♀ decreased in weight for about 35 days from 21.7 gm. to a level of 12 to 13 gm. This level was maintained until the amino-acid ration was changed on the 59th day of the experiment; that is, for a period of over 3 weeks the weight of this mouse was maintained, though it was evident from its appearance that it was not in a good nutritive condition. On the 59th day of the experiment the amino-acid ration was changed to one containing 4 per cent of a mixture of only eleven amino-acids; *i.e.*, lacking glycocoll, alanine, proline, and lysine, as well as serine and oxyproline. The effect of this change was well marked on Mouse 33 ♀, which declined in weight continuously until its death on the 80th day of the experiment, at a weight of 10.1 gm. The total loss in weight of this mouse amounted to 53 per cent of the initial weight. However, the change in ration had no perceptible effect on Mouse 33 ♂, which continued its decline in weight at a uniform rate until its death on the 85th day of the experiment. The final weight of this mouse was 10.5 gm. and the total loss in weight during the entire experiment, 57 per cent of the initial weight. On the 77th day of this experiment the amino-acid ration was changed to one containing only ten amino-acids, as indicated on the chart.

A second feeding experiment, illustrated by Chart II, was undertaken with the second amino-acid ration used in the preceding experiment; *i.e.*, the ration containing 4 per cent of a mixture of eleven amino-acids. On this ration, alternated with the non-nitrogenous ration, Mouse 32 ♂ decreased in weight almost continuously (aside from the fluctuations caused by the alternation of rations) from an initial weight of 21.5 gm. to 15.5 gm. on the 35th day. At this point the weight increased rapidly for 4 or 5 days and then decreased suddenly. The mouse was found dead in the cage on the 45th day of observation, and was partially eaten by Mouse 32 ♀. At the time of this sudden increase in weight

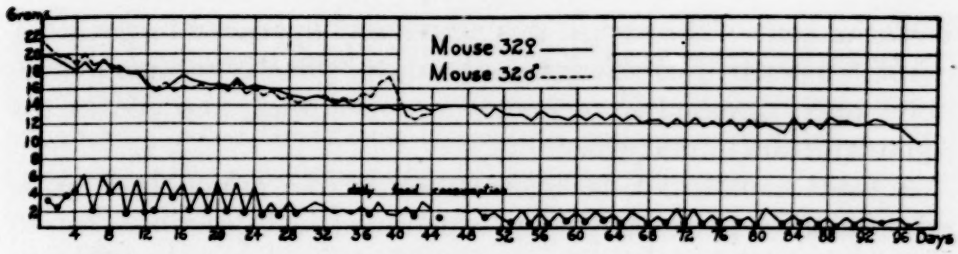

CHART II. The data for Mice 32 ♂ and 32 ♀ are presented in the above curves in a manner similar to the data in Chart I. For the period up to and including the 83rd day the amino-acid ration used contained: 0.25 per cent isoleucine, cystine, tryptophane, arginine, histidine, valine, aspartic acid, and phenylalanine, 0.50 per cent leucine and tyrosine, and 1.00 per cent glutamic acid—a total of 4.00 per cent of eleven amino-acids. From the 83rd day to the end of the experiment the amino-acid ration given contained: 0.25 per cent cystine, tryptophane, valine, histidine, and phenylalanine, 0.50 per cent tyrosine, leucine, arginine, and asparagine, and 0.75 per cent glutamic acid—a total of 4.00 per cent of a mixture of ten amino-acids.

the mouse had a puffy unhealthy appearance, probably due to an accumulation of water in the tissues. During the 45 days of observation this mouse lost 39 per cent of its weight. Mouse 32 ♀ decreased in weight regularly, but at a slower and slower rate; from about the 60th to the 94th day the weight was practically constant as is indicated by the weights tabulated below:

Weight of Mouse 32 ♀.

Day of test.	*gm.*
55	12.4
60	13.0
65	12.4
70	11.8
75	12.2
80	11.7
85	11.5
90	12.3
95	11.7

On the 83rd day of the experiment the amino-acid ration was changed to one containing 4 per cent of a mixture of ten acids, the lack of isoleucine constituting the main difference between this and the preceding ration. On the 98th day of the experiment the mouse died at a weight of 9.8 gm., the total loss of weight during the entire experiment amounting to 50.5 per cent of the

initial weight. The mouse was active and appeared to possess a normal appetite until the last 5 or 6 days, when it became noticeably weak, assumed a very anemic appearance, and contracted some infection in one eye.

A third mouse, No. 50 ♂, was put on the amino-acid ration used for the first 82 days of the experiment just described, fed, as usual, alternately with a non-nitrogenous ration. For 12 days immediately preceding this system of feeding, the mouse had been on an amino-acid ration containing only 2.5 per cent of a mixture of eight amino-acids. Throughout the period of feeding on both of these rations the weight of the mouse declined slowly but steadily. The rate of decline was not apparently affected by the change in feeding instituted on the 12th day of observation. On the 57th day of the experiment the mouse died at a weight of 12.5 gm., having lost 44 per cent of its original weight (22.5 gm.).

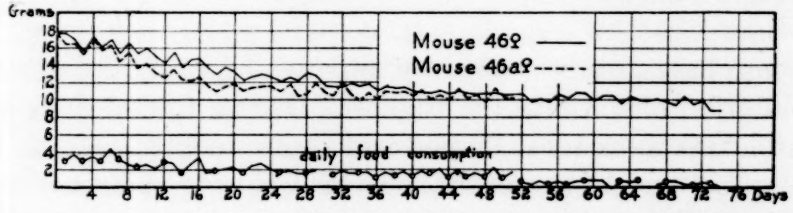

CHART III. The data for Mice 46 ♀ and 46 a ♀ are presented in the above curves in a similar manner to the data in Chart I. The amino-acid ration used throughout the experiment contained 0.25 per cent cystine, tryptophane, valine, histidine, and phenylalanine, 0.50 per cent tyrosine, leucine, arginine, and asparagine, and 0.75 per cent glutamic acid—a total of ten amino-acids, accounting for 4.00 per cent of the ration.

In Chart III are given the results of an experiment on two mice to test the nutritive value of a mixture of ten amino-acids, constituting 4 per cent of a ration that was fed alternately with a non-nitrogenous ration. The proportions in which each amino-acid entered into the former ration are as follows:

	per cent		*per cent*
Glutamic acid	0.75	Cystine	0.25
Tyrosine	0.50	Tryptophane	0.25
Leucine	0.50	Valine	0.25
Arginine	0.50	Histidine	0.25
Asparagine	0.50	Phenylalanine	0.25
		Total	4.00

With this system of feeding Mouse 46 a ♀ decreased in weight from 17.7 gm. to a level of 10 to 11 gm. in a matter of about 30 to 35 days. This level was maintained until the 51st day of the experiment when the mouse was found dead at a weight of 10.2 gm.; that is, for a period of about 3 weeks. Mouse 46 ♀ decreased from an initial weight of 17.7 gm. to a level of 10 to 11 gm. in about 40 days. This level was maintained with insignificant changes until about the 70th day of observation, at which time a rapid falling off in weight occurred until the death of the animal on the 74th day at a weight of 8.9 gm. The following weights of this mouse illustrate the condition of practical maintenance existing from the 40th to the 70th days:

Weight of Mouse 46 ♀.

Day of test.	*gm.*
40	11.0
45	11.0
50	10.6
55	9.7
60	10.0
65	10.0
70	10.2

In this experiment Mouse 46 a ♀ lost 42 per cent of its initial weight, and Mouse 46 ♀ lost almost exactly 50 per cent.

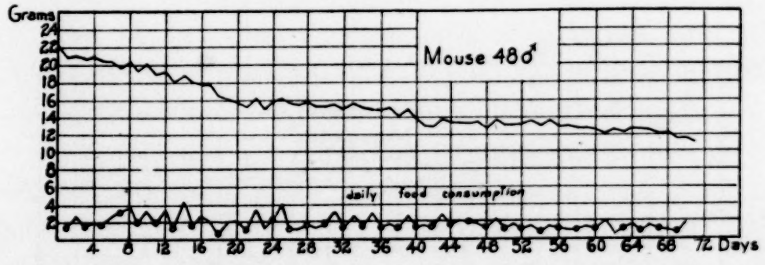

CHART IV. The data for Mouse 48 ♂ are presented in the above curves in a similar manner to the data in Chart I. The amino-acid ration used throughout this experiment contained 0.25 per cent cystine, valine, and histidine, 0.30 per cent tryptophane, 0.45 per cent arginine, 0.50 per cent leucine, asparagine, and phenylalanine, and 1.00 per cent glutamic acid—a total of 4.00 per cent of nine amino-acids. It is to be noted especially that the ration contains no tyrosine.

In the experiments thus far considered, tryptophane, tyrosine, and cystine, the amino-acids known to be indispensable for the maintenance of life, have in all cases been incorporated in the amino-acid mixtures tested. In Chart IV are given the results of a feeding experiment with an amino-acid ration containing 0.25 per cent of cystine, valine, and histidine, 0.30 per cent of tryptophane, 0.45 per cent of arginine, 0.50 per cent of leucine, asparagine, and phenylalanine, and 1.00 per cent of glutamic acid—a total of 4.00 per cent of nine amino-acids, among which tyrosine was not included. On this ration, alternated with the non-nitrogenous ration, Mouse 48 ♂ decreased in weight slowly but continuously from 22.1 gm. to a level of 13.0 to 13.5 gm. in the course of 40 days. This level was maintained for a period of 15 to 20 days, as the following figures show:

Weight of Mouse 48 ♂.

Day of test.	*gm.*
41	13.0
45	13.2
50	13.1
55	13.5
60	12.6

From this time on, the weight decreasd slowly until the death of the animal on the 72nd day. The final weight was 11.0 gm. and the total loss in weight amounted to 50 per cent of the initial weight.

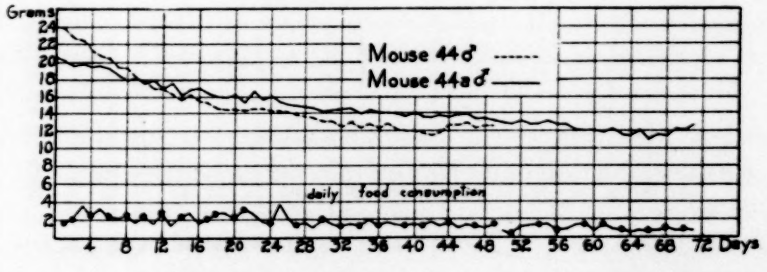

CHART V. The data for Mice 44 ♂ and 44a ♂ are presented in the above curves in a similar manner to the data in Chart I. The amino-acid ration used throughout this experiment contained 0.25 per cent cystine, valine, and histidine, 0.30 per cent tryptophane, 0.45 per cent arginine, 0.75 per cent leucine and asparagine, and 1.00 per cent glutamic acid—a total of 4.00 per cent of amino-acids. It is to be noted that this ration contained only eight amino-acids and, in particular, is lacking in tyrosine and phenylalanine.

A still less complete amino-acid mixture than the above was tested in an experiment the results of which are given in Chart V. The amino-acid ration used in this experiment contained 0.25 per cent of cystine, valine, and histidine, 0.30 per cent of tryptophane, 0.45 per cent of arginine, 0.75 per cent of leucine and asparagine, and 1.00 per cent of glutamic acid—a total of 4.00 per cent of amino-acids. It is to be noted that this mixture contains only eight amino-acids, and, in particular, lacks both tyrosine and phenylalanine. On this ration, fed alternately with the non-nitrogenous ration, Mouse 44 a ♂ declined comparatively rapidly from an initial weight of 24.0 gm. to a weight of 11.5 gm. on the 42nd day of feeding. A slight increase in weight preceded the death of this mouse on the 49th day of the experiment at a final weight of 12.6 gm., the total loss in weight amounting to 47 per cent of the initial weight. The decline of Mouse 44 ♂ was much more gradual, though continuous, and death ensued on the 72nd day of observation after a loss of 39 per cent of its initial weight.

The length of time that mice could be kept alive on mixtures of only a few amino-acids not including tyrosine or even tyrosine and phenylalanine, seemed remarkable. The withdrawal of tryptophane, however, appeared to exert a distinctly more detrimental effect. Three mice were fed alternately a non-nitrogenous ration and an amino-acid ration containing 0.25 per cent of cystine, valine, histidine, and phenylalanine, 0.45 per cent of arginine, 0.50 per cent of tyrosine, leucine, and asparagine, and 1.05 per cent of glutamic acid—a total of 4.00 per cent of nine amino-acids, excluding tryptophane. All three mice decreased continuously in weight. One died on the 33rd day after a loss of 32 per cent of its initial weight (22.8 gm.); another died on the 39th day of feeding, having lost 48 per cent of its original weight (20.1 gm.); the third mouse died on the 44th day of observation, the total loss in weight amounting to 44 per cent of its initial weight (22.0 gm.).

In studying the series of experiments just described on the nutritive value of different mixtures of amino-acids, one is struck by the fact that, aside from the mixture from which tryptophane was excluded, no clearly marked differences in nutritive value (with possibly one exception, mentioned above) are revealed as judged by the rate of decline in body weight or by the compara-

tive longevity of the experimental animals. The different mixtures were often strikingly different in general make-up, containing from eight to fifteen amino-acids in varying proportions. This general similarity in body weight variations is the more surprising in view of experiments on the feeding of isolated proteins inadequate for maintenance due to deficiencies in amino-acid content. These studies show that the different proteins exhibit more or less characteristic effects on the body weight of rats and mice. For example, phaseolin leads to a slow decline in body weight, zein to a more rapid decline, and gelatin to a still more rapid decline. As regards the last two proteins, Wheeler has shown[29] in the case of albino mice that with zein one-third of the body weight was lost in 25 days, while with gelatin one-third was lost in 12 days. It is true that the absence of data of the food intake precludes an analysis of the exact significance of such results. Furthermore, Osborne and Mendel[30] have shown that rations containing 2 per cent of proteins qualitatively adequate for maintenance have rather distinct characteristic effects on the rate of decline of rats, those containing edestin, for example, more nearly maintaining rats than those containing gliadin or glycinine. Thus, it is natural to expect that amino-acid mixtures characterized by qualitatively different types of deficiencies should exhibit distinct characteristic effects, and that such effects were not produced in the experiments reported here is of interest.

In speculating on the causes of the decreases in body weight obtained with all of the amino-acid rations, it must be conceded that probably in no case was enough of the amino-acid mixture consumed to really constitute a fair test of its adequacy. Although the favorable effects of feeding the amino-acid rations used in these experiments, not continuously, but alternately with non-nitrogeneous rations, seem evident, it seems to be a fact that this alternation of the two types of ration, while in most cases it assured a sufficient calorie intake, operated against the attainment of a sufficient amino-acid intake, unless this is much smaller than has hitherto been supposed.

It was surprising how the experimental animals could differentiate between rations containing no added amino-acids, and

[29] Wheeler, *J. Exp. Zool.*, 1913, xv, 215.

[30] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 377.

those containing even very small concentrations of amino-acids. Inspection of Charts I to V is sufficient to show that the non-nitrogenous rations were in general consumed to a much greater extent than the rations containing 6 or 4 per cent of amino-acids. Even in the case of rations containing 2.5 per cent of amino-acids the same marked difference in intake has been observed. It seems impossible that this distinction could have been made on the basis of a difference in taste.

In order to be in a position to appreciate fully the above experiments, feeding tests were made on seven mice using the non-nitrogenous ration only, containing 28 per cent of the modified "protein-free milk." In all of these tests the mice decreased steadily in weight until death, though the periods of survival were different, as Table I shows.

TABLE I.

*Records of Mice on Non-Nitrogenous Ration.**

Mouse.	Period of survival.	Initial weight.	Final weight.	Total loss in weight in per cent of initial weight.
	days	*gm.*	*gm.*	*per cent*
48 ♀	63	19.9	11.1	44
43 ♂	38	26.1	11.3	57
43 ♀	13	20.0	11.8	41
80 ♂	44	27.6	14.2	49
80a ♂	30	24.4	15.9	35
81 ♂	40	24.9	11.9	52
81a ♂	36	22.6	12.0	47

* This ration consisted of sucrose 10 per cent, starch 34 per cent, modified protein-free milk 28 per cent, lard 10 per cent, and butter fat 18 per cent.

It is evident from the results reported in this table that the periods of survival of the mice on the non-nitrogenous ration were distinctly less than the periods of survival of the mice in the more successful feeding experiments with the amino-acid and non-nitrogenous rations fed alternately. Aside from the exceptional behavior of Mouse 48 ♀, the survival periods of the mice on the non-nitrogenous rations are rather strikingly similar to the survival periods of the mice on the amino-acid ration not containing tryptophane; *i.e.*, 33, 39, and 44 days.

That the longer survival of mice receiving amino-acids in their food as compared with mice receiving no amino-acids was not due to a more liberal consumption of food is shown in Table II, in which the average daily consumption of food per 100 gm. of body weight for successive 10 day periods is given (1) for the amino-acid experiments graphically represented in Charts I to V, and (2) for the feeding experiments with the non-nitrogenous ration just described. In the former case, the averages include both the amino-acid and non-nitrogenous ration, which were of approximately equal energy value.

TABLE II.

The Average Daily Food Consumption per 100 Gm. of Body Weight for Successive 10 Day Periods in Feeding Experiments (1) with Amino-Acid Rations, (2) with the Non-Nitrogenous Ration Only, and (3) with an Asparagine Ration. Results Expressed in Gm.

10 day period.	Amino-acid experiments.					Non-nitrogenous experiments.				Asparagine experiments.	
	33 ♂ and 33 ♀	32 ♂ and 32 ♀	46 ♀ and 46a ♀	48 ♂	44 ♂ and 44a ♂	48 ♀	43 ♂ and 43 ♀	80 ♂ and 80a ♂	81 ♂ and 81a ♂	82 ♂	84 ♀
1	6.5	9.9	9.5	11.5	6.1	10.6	9.7	14.6	16.9	9.2	8.3
2	7.9	11.0	8.8	12.2	6.9	11.6		15.6	15.8	8.5	9.1
3	8.1	8.3	8.0	12.2	7.3	8.1	9.3	13.5	14.2	8.9	5.4
4	8.1	7.0	7.1	13.5	5.6	9.5		11.1		7.6	4.7
5	6.9	9.0	7.6	13.5	5.0	7.3				9.0	
6	6.6	9.6	5.9	9.8	8.5	8.6					
7	5.2	8.0	5.7	10.5	7.2						
8	5.8	9.3									
9		8.9									
10		5.3*									

* Average for 8 days only.

In the last two columns of Table II are given the average daily food consumption for two mice on a ration containing 10 per cent asparagine, 10 per cent sucrose, 24 per cent starch, 28 per cent modified protein-free milk, 10 per cent lard, and 18 per cent butter fat. On this ration Mouse 84 ♀ lived for 44 days, losing 56 per cent of its original weight, while Mouse 82♂ lived for 57 days, with a total loss of 50 per cent of the initial weight.

It can hardly be said that the asparagine ration was more successful than the non-nitrogenous ration as regards the period

of survival. However, it seems fairly conclusive that the amino-acid experiments as a whole were much more successful in this regard than the experiments involving the feeding of the non-nitrogenous ration, and that this difference in nutritive efficiency cannot be explained by a more liberal consumption of food in the amino-acid experiments; in fact, the reverse was more truly the case. It seems, therefore, that many of the amino-acid mixtures fed were utilized by the mice in some way in preserving intact the normal functioning of the tissues, possibly, as has been suggested, by furnishing the necessary precursors for the elaboration of the active constituents of the internal secretions, even though such mixtures were not sufficiently complete to make possible the synthesis of protein. These results, therefore, confirm the belief, rapidly gaining ground, that some if not all of the amino-acids occurring in combination in the protein molecule have specific functions in the animal economy aside from the function of serving simply as structural units in the elaboration of the body proteins.

According to the generally accepted theories of protein metabolism, a protein inadequate for maintenance, such as zein or gelatin, that is, a protein totally deficient in one or more of the amino-acids that are necessary for the construction of body protein because it cannot be synthesized by the body, would be of no use in metabolism except as a source of energy. However, McCollum[31] has shown that the pig

> "can utilize the nitrogen of zein very efficiently for repair of the losses due to endogenous or tissue metabolism. The average utilization of zein nitrogen was about 80 per cent, for gelatin 50 to 60 per cent. No evidence was obtained of the formation of additional body tissue from zein, even when the latter was fed in great excess over the maintenance needs of the animals."

In other words, assuming a constant endogenous metabolism, these percentages of the nitrogen of zein and gelatin were retained by the animals and apparently utilized for some purpose other than the construction of protein tissue. Similarly, Henriques and Hansen[32] have shown that the protamine clupein, consisting

[31] McCollum, E. V., *Wisconsin Agric. Exp. Station, Research Bull. 21*, 1912.
[32] Henriques and Hansen, *Z. physiol. Chem.*, 1906, xlix, 113.

of arginine to the extent of about 82 to 83 per cent, was inadequate for maintenance of the white rat, but that its nitrogen was, nevertheless, utilized to some extent in metabolism. Recently Abderhalden[4] has reported experimental results tending to show that, if mice are subjected to protein starvation, while receiving plenty of the non-nitrogenous nutrients, and if in an immediately following period, alanine or glycocoll is added to the basal ration, these amino-acids effect a reduction of the nitrogen loss of the first period, indicating a specific utilization of these nitrogenous nutrients, even when fed singly.

Similarly, the results of the experiments of this paper show that mice survive longer on the feeding of incomplete mixtures of amino-acids added to a basal ration of non-nitrogenous nutrients, than on the feeding of the non-nitrogenous nutrients alone. The most successful experiments of the former type extended over periods 75 to 100 per cent longer than the more representative experiments of the latter type. It appears, therefore, that the requirements of the animal organism for amino-acids resemble its requirement for inorganic ions, in that for the maintenance of life over long periods of time a complete assortment both of amino-acids and mineral elements is essential, while at the same time each essential amino-acid and mineral element has a specific function in metabolism. Thus, incomplete mixtures of either amino-acids or mineral elements will partially cover the body's requirements in the proportion of their completeness, though the elaboration of new tissue, and probably the efficient repair of old tissue, will be impossible in the absence of only one of the essential constituents.

SUMMARY AND CONCLUSIONS.

1. Experiments are reported in this paper in which mice have been kept alive for 70 to 98 days by feeding alternately (1) rations containing 4 to 6 per cent of various mixtures of isolated amino-acids, 6 to 4 per cent sucrose, 34 per cent starch, 28 per cent protein-free milk (prepared either according to the procedure of Osborne and Mendel or to a modification of this procedure described in the text), 10 per cent lard, and 18 per cent butter

fat, and (2) a ration containing 10 per cent sucrose, with other constitutents in the same proportion as in the first mentioned rations. In many of these experiments periods of 15 to 35 days' duration have been observed in which the mice practically maintained their weight.

2. The alternate feeding of an amino-acid ration and a non-nitrogenous ration (except for the nitrogen present in the protein-free milk) induced a better total consumption of food than feeding with an amino-acid ration alone, and in all other respects led to more successful results. However, it is probable that in no case was the amino-acid intake sufficiently large to assure a fair test of its adequacy.

3. Amino-acid rations containing no added tyrosine, or no added tyrosine and phenylalanine, did not give appreciably different results from rations containing these amino-acids. However, if tryptophane was absent from an amino-acid ration, the period of survival of mice fed this ration alternately with the non-nitrogenous ration was noticeably shorter than the periods of survival of mice kept on rations containing added tryptophane.

4. Mice could be kept for much longer periods of time on rations containing mixtures of amino-acids, including tryptophane, fed alternately with the non-nitrogenous basal ration, than when fed the basal ration alone. Furthermore, this difference in survival cannot be accounted for by a difference in energy intake. This fact has been interpreted as meaning that at least some of the amino-acids have specific functions in metabolism aside from that of serving simply as material for the synthesis of body protein. Other evidence from the literature is cited in support of this view, which is substantially the same as that recently and tentatively put forward by Osborne and Mendel.

In conclusion I wish to acknowledge the general supervision of this work by Professor H. S. Grindley, and the aid which his suggestions have contributed. The assistance of Mr. Anton Prasil and Mr. R. A. Nelson in the work of preparing and synthesizing the amino-acids used, is also appreciated.

THE DIGESTIBILITY AND UTILIZATION OF EGG PROTEINS.

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In 1898 Steinitz noted that the ingestion of raw egg-white by dogs was followed by vomiting and diarrhea. This interesting observation appears to have passed almost unnoticed, although no other native proteins are known to give rise to such digestive disorders. When these same facts were observed by Mendel and Lewis in 1913, their possible bearing on the wide spread use of raw eggs in various diets, especially by the sick, prompted the following study.

The Behavior of Egg-White in the Alimentary Tract.

Experiments with Dogs.

Native Egg-White.—Uncooked egg-white fed in any large quantity to dogs invariably caused diarrhea of more or less severity. The feces were soft, pasty, or liquid depending upon the amount of material ingested and upon the individual susceptibility of the subject. The more liquid stools were light in color, and of very offensive odor. Many of them contained much mucus, and residues of unchanged egg-white and of other foodstuffs. Upon standing the surface dried rapidly so as to assume a glazed or varnished appearance. Water extracts of the feces, when boiled, yielded varying amounts of coagulated protein material. The coagulation temperature varied from 68–73°. When an equal volume of saturated ammonium sulfate solution was added to the extract, the latter became turbid, but the grayish precipitate contained only a trace of protein. The addition of acid or of more saturated ammonium sulfate solution to the filtrate caused

the formation of heavy precipitates which acted like ovalbumin. The substance, however, could not be obtained in crystals. The protein in the feces, then, appeared to be unchanged egg-white. The stools of firmer consistency contained less coagulable material than those which were liquid or semi-liquid. Even the apparently formed stools sometimes yielded small amounts of protein upon extraction. In this connection Tsuchiya's (1909) statement that albumin is never found in real formed feces but is usually associated with diarrhea is pertinent.

After eating meals containing enough raw egg-white to induce diarrhea, the subjects usually lost weight to the extent of from 0.25 to 0.6 kilo, or from 3 to 8 per cent of the body weight. Some of the dogs remained stationary in weight, but none showed an increase in this respect.

Vomiting only rarely occurred in consequence of ingesting native egg-white and then only after the larger amounts. The ejected material was practically unchanged by its stay in the stomach.

In a few cases small intestinal hemorrhages were noticed, pointing to irritation of the intestinal mucosa. This occurred only after several days' feeding of the raw food or after much straining at stool.

These abnormal conditions ceased promptly upon substituting meat or "dog biscuit" for the egg-white and could be called forth again by the reverse. In no case was there any "hang-over" effect. Indeed the cessation of egg-white meals was usually followed by constipation. The digestive disturbances which result from feeding dogs native egg-white are, therefore, caused directly by this substance.

Urine, obtained by catheterization on those days when the animals had diarrhea, usually gave negative results when tested for albumin; but occasionally small amounts were found present. These samples of urine also contained very little indican, due no doubt to the rapid emptying of the gut.

Amount of Egg Necessary to Produce Diarrheal Symptoms.—The effects produced by the egg-white were roughly proportional to the amounts ingested. With dogs of 5.5 to 7 kilos in weight the white of one egg causes no or little effect, two eggs may cause softening of the feces, three cause marked softening, and four or

five induce more or less severe diarrhea. The last symptom, then, is brought about by the ingestion of 1 to 1.5 gm. of native egg-white per kilo of body weight. This figure is lower for the heavier animals since dogs weighing from 10 to 12 kilos may develop diarrhea by eating the whites of five to seven eggs. There is much variation in the sensitiveness of the subjects, however; for a large dog may be more easily affected than a small one by the same amount of protein.

Other Factors.—Diarrhea followed the ingestion of: (1) native egg-white unmixed with other foodstuffs; (2) native egg-white beaten with milk; (3) raw egg-white thoroughly mixed with cracker-meal and lard; (4) raw egg-white thoroughly mixed with cracker-meal and lard and well flavored with extract of beef. The first two types of meal produced the laxative effect more quickly than the other two, the stools being passed in 8 to 12 and 12 to 20 hours respectively. The presence of a secretagogue such as meat extract in fairly large amount had no effect on the time of appearance or on the severity of the diarrhea. When a meal containing the whites of four or five eggs was divided into three portions which were fed to the dog several hours apart, the abnormal stool was later in appearing than when the meal was eaten all at the same time, and the diarrhea usually not so extensive. Conversely, if three of four egg-whites were fed at each meal several liquid stools were passed each day.

Tolerance.—It was noticed that when the raw protein was fed for several days in succession its ill effects gradually waned. The time necessary for the abatement of the diarrheal action varied from 3 to 5 days. If then the ingestion were continued, there succeeded a period of alternate days of diarrhea and constipation after which time the egg ceased to exert any marked action. The tolerance thus developed lasted for a short time even after the feeding of the uncooked material was discontinued. These facts are brought out in the following typical protocol.

Dog 1, a healthy terrier bitch weighing 6.52 kilos, received every day a meal containing the whites of four raw eggs well mixed with 60 gm. of cracker-meal and 20 gm. of lard. On the 1st day this caused severe diarrhea with a fall in weight to 6.31 kilos. On the 2nd day the diarrhea continued and the animal vomited some of the meal—one of the rare cases when this happened. The weight fell to 5.96 kilos. The stool on the 3rd

day consisted of a smaller amount of semi-liquid feces. No further vomiting occurred but the weight fell to 6.9 kilos. On the 4th day the pasty feces were much smaller in bulk while the weight rose slightly. This improvement was followed on the 5th day by rather bad diarrhea but the weight was not changed and after this time there was no further loss in weight. The feces of the 6th day were small in amount and pasty. On the next 2 days no feces were passed. The 9th day's stool consisted of a large amount of fairly formed feces. On the 10th day no fecal matter was passed. The quantity of egg given was now increased to five whites but notwithstanding this the next 2 succeeding days showed well formed feces. On the 13th day the stool was small and somewhat pasty while it was well formed again on the next day. At this point the subject was put on a meat diet for 2 days. Then a meal containing the whites of five eggs failed to cause diarrhea. After 2 days more of meat diet, however, the whites of four eggs in the meal brought forth a liquid stool. In one subject this period of induced "immunity" lasted for 12 days.

This tolerance recalls that acquired by the dogs subjected to peritoneal injections of raw egg-white as observed by Cramer (1908). He found that the protein was partly used and partly excreted in the urine. The quantity utilized rose gradually after repeated injections. Hamburger (1902) and Oppenheimer (1904) have observed this as well. That all proteins do not appear in the urine after injection intraperitoneally was shown by Mendel and Rockwood (1905) who found that edestin and excelsin were not excreted by the kidneys after introduction in this way.

Utilization.—The finding of unchanged egg-white in the diarrheal feces showed this foodstuff to be poorly utilized. The extent to which it was used was roughly shown by extracting the 24 hour feces with cold water, boiling this extract, and drying the coagulated material. The feces were made easier to handle by adding bone-ash to the meals and the feces of one period were marked off from those of another with lamp-black. After the ingestion of four to five egg-whites containing 15 to 19 gm. of protein, from 30 to 50 per cent of this could be recovered as coagulum. The latter, of course, contained varying amounts of occluded substances. More exact estimates of utilization were furnished by determining the amount of nitrogen in the feces by the Kjeldahl method. This is open to the objection that a portion at least of the fecal nitrogen is not derived from the undigested foods but is contained in unadsorbed material of the intestinal secretion and in epithelial cells from the intestinal tract.

According to Hammerl, Kermanner, Moeller, and Prausnitz (1897) and Tsuboi (1897) a large part of the nitrogen in the feces comes from these sources. Still another portion may be derived from the organisms living in the alimentary canal, for Osborne and Mendel (1914) have shown that a not inconsiderable part of the feces may consist of bacterial residues. But while the method does not show the exact amount of material digested, the figures so obtained do serve to show the relative degree of utilization. The following protocols are typical.

TABLE I.

Utilization of Raw Egg-White by Dog 1.

Day	Food.	Nitrogen.		Utilization.
		In food.	In feces.	
		gm.	*gm.*	*per cent*
1	Meat.....	2.50	0.13	94.7
2	"	2.50	0.12	95.2
3	"	2.50	0.13	94.8
4	Raw egg-white.....	2.42	1.22	49.8
5	" " "	2.42	1.07	56.0
6	Meat.....	2.50	0.17	93.1

TABLE II.

Utilization of Raw Egg-White by Dog 2.

Day.	Food.	Nitrogen.		Utilization.
		In food.	In feces.	
		gm.	*gm.*	*per cent*
1–3	Meat.....	9.04	0.33	96.3
4	Raw egg-white.....	3.16	1.20	62.0
5	" " "	3.18	1.28	59.5

TABLE III.

Utilization of Raw Egg-White by Dog 4.

Day.	Food.	Nitrogen.		Utilization.
		In food.	In feces.	
		gm.	*gm.*	*per cent*
1	Raw egg-white.....	3.21	1.06	69.0
2	Meat.....	3.05	0.13	95.8

TABLE IV.

Utilization of Raw Egg-White by Dog 6.

Day.	Food.	Nitrogen.		Utilization.
		In food.	In feces.	
		gm.	*gm.*	*per cent*
1	Meat.....	3.06	0.11	97.2
2	Raw egg-white.....	2.97	1.23	58.6

Native egg-white, then, was poorly made use of by these dogs, since from 30 to 50 per cent of the amount ingested was wasted by being ejected with the feces. When Mendel and Lewis (1913) fed this material to dogs only about half of the nitrogen in the meals appeared in the urine for the following 24 hours as contrasted with almost all when meat, casein, edestin, and other proteins replaced the egg-white. Steinitz (1898) reported unchanged egg-white in the feces in considerable quantity but does not state the figures. Vogt (1906) and Falta (1906) found that the nitrogen in uncooked egg-white superimposed upon a standard diet was excreted much more slowly in the urine than that ingested in the form of other proteins. The former ascribes this to a slower rate of digestion and the latter to the longer time necessary to catabolize the larger cleavage products which he supposes adsorbed. Both explanations are fundamentally the same—the native egg-white resists digestive processes.

After the dogs had grown to tolerate the egg-white better, the utilization was better as well. For example, the feces passed by the subject described in the protocol under "tolerance" on the 9th and 12th days were analyzed for nitrogen with results shown in the following table.

TABLE V.

Improvement of Utilization with Tolerance.

Day.	Food.	Nitrogen.		Utilization.
		In food.	In feces.	
		gm.	*gm.*	*per cent*
9	Raw egg-white.....	2.46	0.39	84.3
12	" " "	3.15	0.42	86.7

Dried Egg-White.—The material was prepared by drying native egg-white in the air at temperatures below 50°. 15 to 20 gm. of the dry powder dissolved in water and fed in the usual meal of cracker-dust and lard were as active in causing diarrhea as the raw egg-white itself. This was also true with the subjects of Mendel and Lewis who consider the dried egg-white dissolved in water as equivalent to the original material. Neither was the utilization of the egg-white improved by drying. Several samples were kept at a temperature of about 40° for periods varying from 3 to 7 days, but this again made little difference in their digestibility. Falta (1906) made experiments using desiccated material and found in three cases a utilization of 80 per cent, 80 per cent, and 61 per cent respectively, while the same dogs used almost 100 per cent of casein and gelatin. The method used by Falta was that of superimposition. The feces were usually not analyzed but one dog was noted as having diarrhea. LeClerc and Cook (1906), in the course of work on another topic, fed to a dog meals containing dried albumin, cracker-dust, and lard together with sodium phosphate. They give the following figures:

TABLE VI.

Nitrogen Balance on Diet Containing Egg-White (LeClerc and Cook).

Experiment.	Nitrogen.				Utilization.
	In food.	In urine.	In feces.	Balance.	
	gm.	*gm.*	*gm.*	*gm.*	*per cent*
1	16.72	9.55	9.90	−1.73	40.8
2	16.71	10.89	6.18	−0.37	63.0
3	17.52	6.62	13.85	−3.05	21.0

For the first and third experiments practically all the nitrogen was supplied in the form of egg-white, while for the second, half was in this form and half in egg-yolk. The differences in the balance and utilization are striking. On the last day it will be noted that the nitrogen in the feces was over twice that in the urine. These results may be compared with those given later in this paper.

To determine what effect standing might have on the dried egg-white, experiments were made with commercial albumin

which had been in the laboratory for some years. This caused diarrhea although not so severe as that induced by either the native or freshly dried protein. Its activity was not further diminished by being exposed to bright light for 2 months. The dogs showed considerable ability to overcome the effects of this substance. The following describes a typical case.

Dog 4 was a healthy terrier bitch weighing 6.3 kilos. Its daily meal consisted of 60 gm. of cracker-dust, 20 gm. of lard, and 15 gm. of commercial egg-albumin dissolved in 150 cc. of water. On the 1st day severe diarrhea developed and the utilization of the protein was only 76 per cent. Next day the diarrhea was much less extensive but the weight of the subject had fallen to 6 kilos. On the 3rd day no feces were passed but the quantity on the 4th day was large while the consistency was pasty. The utilization was now 82 per cent. On the 5th day there was only a small quantity of feces and this was true of the 7th day as well. On the alternate days—6th and 8th—the feces were large in quantity and on the last named day better formed. The utilization had risen to 86.5 per cent. The native egg-white and the commercial product act, then, in much the same manner.

In still other experiments the dried egg-white was fed without previous hydration or solution, only enough water being added to the meal to make it eatable. In these cases diarrhea did not generally occur. Water was left in the cages but no more was taken than usual. The quantity of urine was diminished and indican was usually present in notable amounts. The feces were delayed, sometimes not being passed until 23 to 25 hours after the meal. When finally passed they were soft, unformed, of strong odor, and contained much mucus. The utilization was better than in the previous experiments, since it was generally above 80 per cent. In a few cases, however, diarrhea did result from dried egg-white fed in this way.

Cooked Egg-White.—When dogs ate the well cooked whites of four to six eggs they did not display the ill effects attending the use of the same quantities of uncooked egg-white. Moreover, the cooked material was as effective in stopping the diarrhea caused by the raw eggs as was meat. The utilization was very good, being in the neighborhood of 90 per cent, as shown below. The marked difference in the metabolism of the same substance cooked on the one hand and raw on the other is brought out in the following tables.

TABLE VII.

Nitrogen Balance of Dog 1.

Day.	Meal.	Nitrogen.				Utilization.
		In food.	In urine.	In feces.	Balance.	
		gm.	*gm.*	*gm.*	*gm.*	*per cent*
1	Cooked egg-white, cracker, lard.....	3.76	3.20	0.33	+0.23	91.2
2	"	3.76	2.91	0.39	+0.46	89.6
3	Raw egg-white, cracker, lard.....	3.81	3.10	1.87	−1.16	51.0
4	"	3.74	3.27	1.31	−0.84	62.2
5	Cooked egg-white, cracker, lard.....	3.76	3.15	0.43	−0.18	88.5

TABLE VIII.

Nitrogen Balance of Dog 5.

Day.	Meal.	Nitrogen.				Utilization.
		In food.	In urine.	In feces.	Balance.	
		gm.	*gm.*	*gm.*	*gm.*	*per cent*
1	Cooked egg-white, cracker, lard.....	4.32	3.95	0.43	−0.06	90.0
2	Raw egg-white, cracker, lard.....	4.36	3.17	1.81	−0.72	58.5
3	Cooked egg-white...	4.32	4.00	0.44	+0.12	89.8

It will be seen that the cooked egg-white was not so well utilized as meat. For this two factors other than the nature of the foodstuff may be responsible. One is, that the dogs were fed generously which led as usual to more waste; and the other is the presence in the meal of bone-ash, for Mendel and Fine (1912) have demonstrated that even small amounts of indigestible substances in the food cause a poorer use of protein. Kolpakcha (1888) found egg-white to be excellently utilized by dogs—95 per cent or better—even when large amounts were ingested. Although not specifically stated so, the material was probably cooked. Steinitz (1898), on the contrary, states that considerable quantities of coagulated egg-white were passed in the feces of dogs. The differences may possibly be due to variations in

the fineness of division since if dogs are allowed to bolt large pieces of hard-cooked egg-white, fragments of the same can generally be distinguished macroscopically in the feces.

The Temperature Necessary to Improve the Digestibility of Egg-White.—The above results show that simple heating of the native egg-white renders it more digestible and affords the organism a chance to make better use of it. At what temperature is this change effected? To determine this the egg-white was heated in a double-boiler for 30 to 45 minutes with constant stirring. At temperatures below 55° the substance suffered little impairment of its activity in causing diarrhea. This activity is, however, considerably decreased by heating at 55° or 60°. After being subjected to a temperature of 65° for half an hour the digestibility of the egg-white is still further enhanced while at 70° or above, the foodstuff becomes entirely innocuous. Above 55°, more or less coagulation takes place depending upon the degree of alkalinity of the egg-white, while at 70° the protein is entirely coagulated but is very soft and jelly-like in texture.

Experiments with Rats.

The subjects were large, healthy, white rats kept in sanitary cages and supplied plentifully with food and water. The meals consisted largely of separator-milk powder ("Klim") mixed well with varying amounts of egg-white, bread-meal, and lard.

When the diet contained 20 per cent of raw egg-white the feces of the subjects were not different from those of controls fed on a mixed diet although occasionally the presence of small amounts of coagulable protein could be demonstrated. When the proportion of egg-white was increased to 40 per cent half of the animals had softened feces, the scybala being larger and longer than normal. Such stools as these always contained unused protein. All the subjects up to this time gained in weight and maintained excellent condition. Tolerance for the unusual foodstuff was quickly gained since after 7 to 10 days' feeding the feces could not be distinguished in appearance from those of the controls.

Next, a new lot of animals were first fed a diet containing 60 per cent of raw egg-white. This caused diarrhea which in some cases was severe. In the latter contingency the body weight

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decreased while the less susceptible animals, on the contrary, gained somewhat or remained stationary. The food was not relished so that the diminished intake played some part when weight was lost. The ill effects of the native egg-white were still more marked when the proportion in the food was increased to 70 per cent. Several subjects refused to eat after a few days, moping in the cages and neglecting to clean themselves. The feces now became quite liquid at times and often contained mucus. When the fecal matter dried it appeared as though varnished. In a final experiment the rats were fed a mixture of 80 per cent raw egg-white and 20 per cent milk powder of which they partook rather sparingly. They lost weight rapidly, all were afflicted with diarrhea, and after 10 days it was evident that their health was much impaired. Six of these animals while having diarrhea were killed with chloroform and the intestines examined. In half the cases everything was normal while the degree of intestinal inflammation perceived in the other three did not appear significant.

The use of cooked instead of raw egg-white was followed by no untoward effects. All the rats gained in weight and remained well and active even when the diet contained a large proportion of the coagulated protein.

Falta and Noeggerath (1905) fed rats a diet in which dry egg-white was the only protein. The animals did not live longer than 94 days in the most favorable cases. The weight steadily declined and death followed when the weight fell to two-thirds or three-fifths of its original value. The rats suffered from conjunctivitis and other eye diseases. Knapp (1908) reports similar results. All his rats experienced diarrhea and conjunctivitis. One soon died, the others following in from 17 to 24 weeks, after losing much weight. Maignon (1912) fed rats exclusively on egg-white, either raw or cooked, which was found entirely inadequate to sustain them. Weight was quickly lost in spite of the increased quantities of food ingested. Death occurred after various periods when the body weight had been much reduced—in some cases as much as 40 per cent. Frank and Schittenhelm (1912) also failed to nourish rats when the only protein furnished was egg-white.

Von Knieriem (1885) fed mice exclusively on dry egg-white. They could not maintain existence and some succumbed in 3 days. Röhmann (1914) used mice as subjects, feeding them mixed diets some of which contained egg-white. He concluded from the results of his experiments that this substance sustained life poorly in comparison with other proteins.

All the untoward effects noted by these investigators cannot be ascribed to the egg-white but more justly to the lack in the diet of important food accessories such as the vitamines. Osborne and Mendel (1911) kept rats growing well on rations containing cooked egg-white as sole protein for 170 days and the ensuing decline was arrested, not by changing the protein but by adding a small amount of butter fat. As regards the eye troubles, rats are prone to suffer from epidemics of conjunctivitis and other diseases if poorly nourished or kept in unsanitary surroundings. Both Falta and Noeggerath and Knapp experienced these troubles with other proteins in the diet than egg-white; but they appeared to be milder and more tractable in the former case.

Experiments with Rabbits.

The experimental animals were kept in metabolism cages and liberally supplied with mixed food. The raw egg-white was fed by means of a stomach sound.

1. Two rabbits each received the whites of two eggs. One passed a small amount of semi-liquid matter while the feces of the other and of a control which received an equivalent quanity of water were normal.

2. Two rabbits each were fed the whites of three eggs. One had moderate diarrhea while the feces of the other were pasty and increased in quantity above those passed on the mixed food.

3. Three rabbits each were given the whites of two eggs at 10 o'clock and the whites of two more at 2 o'clock. Two were afflicted with diarrhea of some severity. One of them ate most of the fecal matter in preference to food. The third animal passed a large amount of very pasty feces, the scybala being much larger than normal.

Von Knieriem (1885) fed three rabbits each a meal containing:

	gm.
Dry egg-white	15
Meat extract	5
Sugar	10
Horn shavings	2
Water, cc	225

The resulting feces were very soft and abnormal. Utilization of the egg-white in the three cases was calculated to be 61, 67.2, and 70.2 per cent respectively. The character of the feces and the poor utilization of the protein were doubtless due in part to

the presence in the food of the horn shavings which acted in the same manner as the indigestible substances used in the experiments of Mendel and Fine and Mendel and Lewis. Nevertheless, von Knieriem decided that egg-white was, in contrast to almost all other proteins, poorly digested. Mendel and Rose (1911) found it impossible to feed any large amount of native egg-white to rabbits without inducing diarrhea.

Experiments on Man.

Experiments were made with native egg-white and raw eggs upon a number of persons, and the cooperation of still others who were eating raw eggs in order to gain weight was secured. The data collected confirmed the points already brought out. Most of the subjects experienced diarrhea which generally abated after several days' ingestion of the raw foodstuff, and many complained of intestinal discomfort. The material was poorly utilized. These experiments will be discussed more in detail together with the question of the use of raw eggs in practical dietetics, in another place.

The results of these experiments are in accord with those of other investigators. Falta (1906) found raw egg-white to be utilized by man to the extent of 70 per cent. A still lower value, 50 per cent, was reported by Wolf (1912) who has described the peculiar behavior of this foodstuff when ingested by man. He did not observe diarrhea but the feces were of soft consistency. When raw egg-white was in the diet there was over twice as much nitrogen in the feces as when the food contained the same quantity of nitrogen in the form of other proteins. On one occasion the feces contained more nitrogen than did the urine. The latter did not contain albumin, a result in contrast to those of Hamburger (1902) and Cramer (1908) who claimed that albuminuria followed the ingestion of large doses of native egg-white by man. Previously Oertel (1883) had not been able to find albumin in the urine in such cases, and neither did uncooked egg-white cause an increase in excreted albumin in preexisting albuminuria. Wolf also noted that when either raw or cooked egg-white was superimposed upon a standard diet there was a delay in the excretion of nitrogen and sulfur, the delay being greater

in the sulfur elimination. He suggested that the sulfur complexes in the uncoagulated egg-white have considerable power to withstand the action of the digestive enzymes.

Explanations of the Behavior of Egg-White in the Alimentary Tract.

The Effect of Heating on Egg-White.

The improvement in the digestibility of raw egg-white effected by heating may be brought about by the increased temperature in four different ways. These are: (1) The temperature used in cooking kills any bacteria present; (2) heating changes the physical texture of the egg-white; (3) heating destroys enzymes normally found in this native protein mixture; (4) heating changes the chemical nature of the egg-white.

1. Heating Kills Bacteria.—That bacteria in the egg-white cause the diarrhea attending its use is improbable. It is true that Wiley (1908), Lamson (1909), Pennington (1910), Maurer (1911), and Stiles and Bates (1912) have reported a large percentage of the eggs examined by them infected. But the more recent work of Rettger (1913), who carried out far-reaching tests with the greatest care to prevent accidental contamination, led him to the opposite conclusion. Of the whites of 582 fresh eggs only seven, or 1.2 per cent, showed the presence of bacteria and some of these cases were unquestionably due to contamination. These results are supported by those of Horowitz (1902) and confirmed by Kossowicz (1913). And not only is egg-white usually sterile but it has strong antiseptic properties and in many cases a marked disinfectant action on bacteria as demonstrated by Laschtschenko (1909) and Rettger and Sperry (1912). The bactericidal properties are destroyed by heating the egg-white to 65–70°. In view of these facts it is unlikely that the unusual behavior of native egg-white in the alimentary tract can be due to bacteria.

2. Heating Changes the Physical Texture.—When raw egg-white is heated above 55° it coagulates and loses its properties as a viscid liquid. It is possible that this change is responsible for the bettered digestibility of the cooked protein especially since, as shown above, this improvement is not brought about by heating at temperatures too low to cause coagulation.

Two sets of dogs were fed meals exactly alike except that one

received native egg-white and the other cooked egg-white ground to a fine pulp. By the addition of water both types of meal were made as nearly of the same consistency as possible. Those meals containing the raw foodstuffs always caused diarrhea while the others did not.

For further evidence on this point there was added to raw egg-white enough sodium hydroxide to prevent coagulation when the mixture was heated even above 70°. By this treatment the egg-white was little altered, only becoming somewhat more viscid; yet when fed to dogs it failed to cause diarrhea.

Other experiments to be discussed later in detail also showed that the egg-protein could be made digestible without changing its fluid character.

While it may be said that the peculiar physical nature of the substance is not *per se* the factor giving rise to diarrhea and poor utilization, the protein does, nevertheless, act while in the stomach in an unusual manner. Beaumont (1833) was the first to discover that raw egg-white leaves the stomach more rapidly than other foods, including cooked egg-white itself. Some objections may be raised against these early observations because of the abnormality of Beaumont's subject; but that the protein does act in the manner described has been confirmed recently by Cannon (1905), and London and Sulima (1905). The former found the egg-white to pass through the pylorus at a rate comparable to that of the carbohydrates which are among the first foodstuffs to enter the intestine after ingestion whereas protein leaves the stomach slowly. The egg-white was the only protein to act in this way among those investigated. London and Sulima by means of intestinal fistulas found that the egg-white left the stomach in large gushes, often faster than the peristaltic waves. During its stay in the stomach it retained its alkaline reaction for some time or passed into the intestine in this condition. After feeding a dog native egg-white these workers recovered 73 per cent unchanged from a fistula in the ileum.

The amount of gastric proteolysis undergone by this protein, already decreased by its short stay in stomach, is still further diminished by another unusual property observed by Pawlow (1902); namely, that the egg-white acts ineffectively in stimulating a flow of gastric juice. It acts in this way only like

so much water. But even this is not the end of the story, for Abderhalden and Pettibone (1912), and Bizarro (1913) state that native egg-white offers considerable resistance to the action of pepsin. If the egg-white is heated, however, and especially if coagulated at 80°, it is more quickly acted upon by the pepsin than when uncooked, and hydrolysis proceeds farther. After the material is once coagulated the rate of digestion is lessened by heating at higher temperatures. The fineness of division of the coagulum also affects the rate of digestion.

We may picture this native protein, then, as quickly leaving the stomach, accompanied by scanty amounts of gastric juice and practically unaltered by pepsin. Perhaps this lack of gastric digestion is at the bottom of the egg-white's unusual behavior as a foodstuff. That it does have a bearing on the subject was shown by feeding dogs partially digested egg-white. The protein together with 0.2 per cent pepsin and 0.2 per cent hydrochloric acid was heated for several hours at 37°. The digestion was continued until the mixture upon neutralization gave a heavy precipitate, in the filtrates from which no coagulable protein could be detected. This material, which was little altered in texture from the original, was as inactive as cooked egg-white in causing diarrhea.

However, Cannon, and London and Sulima have shown that even coagulated egg-white leaves the stomach much more rapidly than other proteins, although less so than when in the native condition. When cooked it does, in contrast to the raw material, call forth an early secretion of hydrochloric acid with which it unites. But the difference in rate of gastric discharge is not enough to account for the differences in digestibility, so one must consider the behavior of the two in the intestine.

As stated above, the native egg-white in the stomach fails to cause a good flow of gastric juice. According to the mechanism which Bayliss and Starling (1902) propose for the action of secretin, it is probable that once in the intestine the raw egg-white meets comparatively little pancreatic juice since the amount of the latter is proportional to the quantity of acid entering the duodenum—a fact observed by Pawlow (1902) as well. Furthermore, Brüno and Klodnizki (1914) showed that no bile was passed into the intestine when raw egg-white was ingested.

Okada (1915) has confirmed this to the extent that in his experiments the secretion of bile was small and seemed to be dependent upon the degree of digestion undergone by the protein in the stomach. Coagulated egg-white always caused a good flow of bile.

3. Heating Destroys Enzymes.—In the intestine the digestion of the egg-white is still further checked by the pronounced resistance it exerts against the action of trypsin. Vernon (1904) found the antitryptic activity to be more marked with this protein than with any other. In solutions containing 0.5 to 1.0 per cent of raw egg-white the trypsin action on fibrin was lowered to only 2.7 per cent of the normal, while even one part of egg-white in 6,000 reduced the activity to 45 per cent of the normal. When the egg albumin was exposed to a temperature of 60° its antitryptic influence was not diminished and it retained a good deal of its inhibitory power even after coagulation at 100°.

The native egg-white, however, is unlike collagen which is not acted upon at all by trypsin; for Bayliss (1908) has shown that if sufficient time be allowed the enzyme will act as completely upon uncooked as upon cooked egg albumin. For some hours after being brought together the trypsin does not change the egg-white in the slightest degree and it requires over 70 hours before the digestion equals that of the cooked material under the same conditions. Hedin (1907), Cohnheim (1912), Abderhalden and Pettibone (1912), Long and Johnson (1913), and Bizarro (1913) have all demonstrated the strong antitryptic effect of egg-white which Hammarsten (1912) considers remarkable.

To explain this behavior the existence of a specific anti-enzyme, antitrypsin, has been postulated which is supposed to unite with the trypsin much as an antitoxin does with its corresponding toxin. A later view is that the trypsin is adsorbed by the colloidal egg-white which thus cuts down tryptic activity in much the same way as did charcoal in the experiments conducted by Hedin (1906, b). Recently Maxwell (1915) has shown that boiled starch adsorbs pepsin and so hinders its activity. That egg-white does act in this manner is suggested by the observations of Delezenne and Pozerski (1903), Vernon (1904), Gompel and Henri (1905), and Hedin (1907) who found that raw egg-white prevents the tryptic digestion of other easily digested proteins.

It has already been stated that native egg-white resists peptic digestion. It is interesting to note that proteolytic enzymes other than those occurring in the intestinal tract are also unable to act upon this substance. The work of Chittenden, Joslin, and Meara (1892), and Chittenden (1894) showed that raw egg-white was much more poorly digested than coagulated egg-white by *bromelin*, the proteolytic enzyme of the pineapple. Jonescu (1907) and Sachs (1907) found that *papain* was practically unable to act upon native egg-white. If the latter was heated or incubated with dilute acids the antiproteolytic action toward papain was destroyed.

If native egg-white contains antitrypsin, then heating may improve its digestibility by destroying the anti-enzyme. Talarico (1910) found that preliminary heating of the egg-white aided tryptic digestion. There was no effect, however, until the temperature reached 70° after which there was an orderly liberation of amino-acids up to 130°. Frank (1911) also found cooked egg-white to be better digested than raw, the optimum temperature being 70–75°. At temperatures above this the digestibility decreased in proportion to the hardness of the coagulated material. Bizarro (1913) considered the best temperature to be 80°. In addition to these observers Abderhalden and Pettibone (1912) and Long and Johnson (1913) found the coagulated egg-white much better digested than the raw. The latter observers state that cooked egg-white, however, is acted upon slowly by trypsin as compared to pepsin.

It is significant that the temperature (70°) which was found sufficient to rob the egg-white entirely of its activity in causing diarrhea is the same as that given above for the optimum activity of trypsin.

Beyond the resistance to trypsin the properties of the alleged antitrypsin in the egg-white are little known except that Vernon (1904) found its action much weakened by heating with dilute sodium carbonate. Hedin (1904) also used sodium carbonate to destroy the anti-enzyme.

The following experiments were made to determine whether the antitrypsin could be the source of the unusual behavior of egg-white.

The whites of five to six eggs were dialyzed for several days.

When fed to dogs this material was effective in causing diarrhea. The antitrypsin, if present, is not diffusible. Cathcart (1904) found that the antitryptic action of blood serum is not removed by dialysis.

Egg-white was incubated with 0.2 per cent sodium or potassium hydroxide or 0.25 per cent sodium or potassium carbonate for several hours at 37°. Fed to dogs the material was no different in behavior than cooked egg-white. This treatment does not alter the texture to any extent.

The native protein was heated in an incubator at 37° for 4 to 8 hours with 0.2 per cent acetic or hydrochloric acid. By this treatment it was rendered digestible and no diarrhea followed its use. This process also left the original texture unaltered.

Jobling and Petersen (1915) succeeded in extracting antitrypsin from blood serum with chloroform. It was found impracticable to extract native egg-white with chloroform or ether since a considerable quantity of the protein was precipitated and the liquids, especially the chloroform, did not separate well. After standing for 12 to 24 hours the mixtures were dried rapidly in a current of warm air at room temperature. The dry material was thoroughly mixed with water and fed to dogs. It did not cause diarrhea. Nothing had been removed from the native egg-white and judging by the work of Jobling and Petersen the treatment was scarcely drastic enough to destroy the anti-enzyme.

Further attempts at extraction were made more successfully by previously drying and powdering the egg-white. In order to make the extraction as complete as possible the mixture of dry egg-white and ether or chloroform was allowed to stand for 4 days, on each of which it was well shaken for a period of 30 to 60 minutes. The extract was then separated and evaporated to dryness at room temperature. The residue from the extract was triturated with water and mixed as well as possible with finely minced, coagulated egg-white. This mixture, assumed to contain any antitrypsin originally in the egg-white, failed to cause diarrhea when fed to dogs. The residues insoluble in chloroform or ether were dried, mixed with water, and fed to dogs with negative results.

It is well known that enzymes in general are soluble in dilute and precipitated by strong alcohol. Egg-white was coagulated with strong alcohol and filtered. If antitrypsin was precipitated by

the reagent it should be contained in the coagulated protein; yet when this was freed from alcohol and fed to dogs it caused no diarrhea. Neither did the filtrate appear to contain antitrypsin, for the residue obtained from it by evaporation well mixed with cooked egg-white was fed to dogs with no untoward results. In still other experiments the egg-white was precipitated as before with strong alcohol. After filtering, the coagulated protein was finely minced, ground up with dilute alcohol, and finally washed with dilute alcohol. By this treatment it might be supposed that antitrypsin, if present in the egg-white, would first be precipitated and then washed out of the finely divided coagulum by the dilute alcohol. Accordingly the filtrates and washings were evaporated until the alcohol was removed and fed to dogs after being thoroughly incorporated with cooked egg-white. In no case was diarrhea induced. Finally the washed coagulum was fed but proved as ineffective as the extracts.

Those of the above experiments, therefore, which were planned to remove an active substance from egg-white—by extraction or washing—appeared to fail since the separated material did not render cooked egg-white indigestible. In contrast, those experiments calculated to make the native egg-white digestible by destroying the anti-enzyme were successful. It may justly be argued, however, that antitrypsin, even if contained in the residues from the extracts, cannot be mixed intimately enough with cooked egg-white to make it really indigestible.

4. Heating Changes the Chemical Nature.—When egg-white is coagulated its chemical nature is altered. This change may be the causal factor in bettering its behavior in the intestinal canal. Changes of like nature brought about by other agents than heat were similarly effective.

Some of the experiments already described bring out this point. Thus egg-white precipitated with alcohol did not affect dogs as did the original protein. It made no difference whether the precipitate was filtered off at once or allowed to stand in contact with the alcohol for several days. In other experiments the egg-white was denatured by the minimum quantity of moderately strong alcohol and the whole mixture dried quickly at a low temperature. This material sometimes considerably softened the feces of dogs by which it was ingested but this may be due to the fact that the treatment usually leaves some of the original protein unaltered.

It has already been stated that dried egg-white extracted with chloroform or ether was readily digested. When these residues, from which little had been removed by extraction, were mixed with water and fed to dogs they did not cause diarrhea. Sometimes, after the extraction had been made with ether, the feces were softer than normal. This lack of activity was not because the reagents extracted any active material as shown above, but apparently because the protein was more or less denatured by them. Before extraction the dried egg-white was entirely soluble in water, but the treatment so changed it that much of it became insoluble.

Egg-white was chemically modified in still another way by converting it into alkali-metaprotein. This was prepared by the gradual addition of potassium hydroxide to native egg-white with constant stirring. The transparent jelly was finely divided and thoroughly washed with cold water. Fed to dogs it proved an excellent foodstuff. The feces formed by it closely resembled the normal feces in being dry and crumbly. Klug (1897) found that pepsin from the stomach of dogs digested alkali-albumin better than any other protein in his list, at the bottom of which he placed coagulated egg-white. In general the same thing was true with pig and ox pepsin. Vernon (1904) considered alkali-albumin prepared from egg-white more easily digested by trypsin than the native protein.

As stated previously, if egg-white is partially predigested by pepsin or incubated with dilute acids and bases it loses its ability to cause diarrhea. All of these procedures, in addition to any effect they may have on antitrypsin, change the chemical nature of the egg-white. It is true that some of these changes are not pronounced, but in this respect the altered substances may be like the racemized proteins of Dakin and Dudley (1913) who found that such a slight chemical change as a partial reversal of the direction of polarization was sufficient to convert casein into an entirely indigestible substance.

Résumé.—From the evidence at hand it is reasonable to assume that the indigestibility of the egg-white is due primarily neither to its physical texture nor to any bacteria present. But it seems impossible to make a decision between the other two explanations offered, the difficulty being that any process affecting one

factor modifies the other as well. Thus, when incubation with acid improves the digestibility of the native protein, we may explain it from the one view-point as due to the destruction of the antitrypsin, and from the other as due to the conversion of the indigestible egg-white into digestible acid-metaprotein. When treatment with chloroform renders the egg-white digestible, we may assume that this is caused either by the removal or destruction of the anti-enzyme, or by the change effected in the chemical properties of the protein.

The simplest assumption is that raw egg-white is attacked with much difficulty by the digestive enzymes. It is not contended that raw starch, agar, collagen, cellulose, and other indigestible substances contain anti-enzymes. Moreover, the existence of such substances has been seriously doubted of late by investigators like Bayliss (1908) and Cohnheim (1912), the latter of whom considers that, "the evidence does not permit us to speak of specific antiferments." Probably the explanation of the behavior of antitryptic substances most in favor at present, is that which postulates an adsorption of the trypsin, the effective concentration of which is thereby reduced.

What Component of Egg-White Is Responsible for Its Peculiar Behavior in the Alimentary Tract.

The Action of the Individual Proteins of Egg-White.

The proteins of egg-white were separated from each other according to the method of Osborne and Campbell (1900). Care was taken to have the various fractions as free from each other as possible and each was dialyzed free from ammonium sulfate before being used. The amounts of the various proteins used were equivalent to those contained in the whites of five or six eggs, which amount of egg-white had previously been found sufficient to cause severe diarrhea in the dogs used as subjects.

Globulin.—According to Osborne and Campbell globulin is present in egg-white to the extent of 7 per cent of the total solids. The whites of five or six eggs contain approximately 1.5 to 2 gm. of globulin. These amounts of freshly prepared, moist globulin mixed with the usual meal did not affect the dogs in the least and the same was true when quantities four or five times as

large were ingested. Even 20 gm. of purified egg globulin prepared 2 years previously did not perceptibly alter the consistency of the feces, although it was not very well utilized.

Ovalbumin.—The ovalbumin was obtained in fine cyrstals. After being dialyzed it was quickly dried at a low temperature. Osborne and Campbell succeeded in crystallizing 50 per cent of the total proteins in egg-white but consider that all the other fractions contain more or less of this substance. While the actual proportion present is, then, somewhat doubtful, for these experiments it was assumed to be 60 per cent. The whites of five or six eggs would then contain 12.5 to 16 gm. of ovalbumin. Fed in these amounts to dogs it caused diarrhea which was more marked when larger amounts were ingested. Its action, however, was not so marked as might be expected from the activity of the native egg-white itself and was also more variable. Thus one dog, for instance, in which diarrhea had been induced several times with raw egg-white failed to react at all to the ovalbumin. Perhaps this is not surprising considering the amount of manipulation necessary to obtain the pure ovalbumin. While the globulin is separating the mixture becomes strongly alkaline from the formation of ammonium hydroxide, and another change—possibly oxidative—is going on as shown by the gradually deepening color of the solution. Later the ovalbumin stands for a long time in contact with acid, is dialyzed for several days, and dried. Osborne and Campbell indeed regard the crystallized substance as different from the original protein and consider that it is probably a combination of the protein with acid.

Conalbumin.—The amount of uncrystallizable albumin in egg-white is not definitely known but it is probably from 25 to 35 per cent of the total proteins. In five or six egg-whites there would be 4.5 to 6 gm. of conalbumin. Fed in these amounts it generally caused the formation of pasty feces, which effect was increased where the amount ingested was larger. A mixture of ovalbumin and conalbumin in the proportion in which they occur in egg-white fed to dogs in amounts equivalent to 85 per cent of the total proteins in five or six egg-whites caused diarrhea.

Ovomucoid.—It seems unlikely that ovomucoid is the disturbing protein of the egg-white since it is present in small amount—10 per cent according to Hammarsten,—is unaffected by heating

in boiling water, and is precipitated unchanged by alcohol, so that it was present in the alcohol precipitates which failed to cause diarrhea. This supposition was confirmed by feeding to dogs ovomucoid prepared as follows. The whites of five or six eggs were diluted with water and the globulin and albumins coagulated by boiling after the addition of a little acetic acid. The filtrate containing the ovomucoid was fed to dogs mixed with the usual meal but did not cause diarrhea.

The albumin fraction, therefore, appears to be the indigestible portion of the egg-white. This is in harmony with the observations of Mendel and Lewis who found that purified ovalbumin fed to dogs in comparatively large amounts caused profuse diarrhea. Vernon (1904) found crystallized albumin to be even more resistant to trypsin than native egg-white. Bainbridge (1911) noted that certain forms of bacteria do not appreciably break down this substance even in the presence of sufficient non-nitrogenous food to insure vigorous bacterial growth. In view of these results it is interesting that the antitryptic action of blood-serum according to Hedin (1904), Cathcart (1904), and Vernon (1908) is associated with the albumin and not with the globulin.

The Digestibility of Duck Egg-White.

Experiments with the white of the eggs of the Eastern Indian Runner demonstrated that it, too, was indigestible, caused diarrhea, and was poorly utilized. The egg-white of the common fowl, then, is not unique in its exceptional conduct as a foodstuff. The egg of the duck appears to have been little investigated but the white differs in appearance from that of the hen's egg, and Panormow (1906) thought that it contained a special protein not found in other eggs.

The Behavior of Egg-Yolk in the Alimentary Tract.

When egg-yolk was fed to dogs in any large quantity as part of a meal containing lard and cracker-dust, it usually caused a rather severe digestive disturbance of which vomiting was the main symptom. The vomitus contained much bile, and frequently comparatively large amounts of this liquid were expelled from the stomach. Diarrhea sometimes accompanied this

vomiting. The subjects were apathetic and had little appetite for several days. These effects were brought about by cooked as well as by raw egg-yolk. The picture of this digestive disorder is thus quite different from that induced by native egg-white.

The regurgitation of bile suggested the fat as the cause of the trouble. Therefore the lard was omitted from the meal with favorable results since no diarrhea or vomiting was then noted. The same end was attained by extracting the fat from the egg-yolk by means of ether. The crude yolk-protein, freed from ether, was fed with lard and cracker-meal to dogs, and was inactive. The dogs affected by egg-yolk in the way described seem, then, to have had a low tolerance for fat. The disturbing effect of the yolk is considerably less than that of the white; for while, in one case, the whites of four raw eggs caused diarrhea the same subject ate six raw yolks with impunity.

The egg-yolk and ovovitellin were found to be excellently utilized and there appears to be nothing in the literature to show that they are indigestible. Mendel and Lewis' (1913) experiments showed the excretion of nitrogen after feeding ovovitellin to be the same as that after meat, a result in opposition to that obtained with coagulated and uncoagulated egg-white where the nitrogen excretion was delayed. McCollum (1909) sustained life in white rats for 18 weeks on nothing but egg-yolk with no unfavorable results, while it may be recalled that Maignon, Knapp, and von Knieriem could not keep dogs, rats, or mice alive on egg-white alone. Laschtschenko (1909) found that bacteria thrived well on egg-yolk, that it does not possess germicidal properties, and cuts down the bactericidal action of the egg-white.

SUMMARY.

Raw egg-white is found to be a decidedly indigestible substance. It may cause diarrhea in dogs, rats, rabbits, and man when ingested in any large quantity. Its utilization by the body is poor since it is used only to the extent of 50 to 70 per cent. Subjects can acquire a certain tolerance for the native protein after ingesting it for several days so that it no longer causes diarrhea and is somewhat better utilized.

Raw egg-white can be made digestible through coagulation